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The diversification of the loach genus *Schistura* McClelland, 1838 (Teleostei, Nemacheilidae) in Sri Lanka

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Abstract

The Palk Isthmus, which connected Sri Lanka to the Indian mainland during periods of low sea levels, has been the only route by which freshwater organisms could enter the island. The endemic aquatic biodiversity of Sri Lanka is dominated by diversifications stemming from founders that immigrated when the Isthmus was both emergent and possessed an aseasonal-mesic climate. The loach genus *Schistura* McClelland, 1838, among the most speciose of freshwater-fish genera, has three known representative species on the island. Based on two mitochondrial and two nuclear gene markers, we investigate the evolutionary relationships of *Schistura* in Sri Lanka, showing it to be a monophyletic diversification. Molecular species delimitation analysis recovered eight lineages (MOTUs). These exhibit strong geographical structure; each confined to one or more contiguous basins and two confined to a single basin. There is evidence for gene flow across watersheds, likely due to headwater river-capture. *Schistura* lineages in the central hills appear to result from multiple independent colonization events. The most widespread lineage in this region likely represents a more recent colonization, following a Late Pleistocene highland extirpation. An analysis of 39 phenetic traits across > 250 specimens revealed marked morpho-molecular incongruence. Taxonomy dependent entirely on phenetic traits is likely to be unreliable in rapidly diversifying populations such as these.

Key Words

Highland colonization, molecular phylogenetics, morpho-molecular incongruence, phylogeography, river capture, species delimitation, systematics

Introduction

The colonization of islands offers interesting opportunities to investigate morphological and genetic diversification following an initial invasion (Garcia-Porta and Ord 2013). As an island lacking a vertebrate fauna that pre-dates the Paleogene, Sri Lanka provides an interesting case for investigating such colonisations, especially because there was only a single route of entry: the Palk Isthmus. Having been emergent during low sea-level periods since at least the mid-Oligocene, it provided the only terrestrial connection, even if only intermittently, with

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the subcontinent mainland (Pethiyagoda and Sudasinghe 2021). Despite the southern region of the Western Ghats of India and the south-west quadrant of Sri Lanka having enjoyed a perhumid climate that supported rainforests during most of this period, the climate of the Isthmus and the adjacent plains in India and Sri Lanka were, except for brief pluvial spells, too dry and seasonal to facilitate the exchange of species associated with mesic habitats. Indeed, there is no evidence for the ingress or egress of biota associated with rainforests since the Late Miocene (Pethiyagoda and Sudasinghe 2021).

The island's perhumid 'wet zone', however, contains several monophyletic faunal diversifications originating from colonizations that took place between the Late Oligocene and Late Miocene: some 80 species in the rhacophorid shrub-frog genus *Pseudophilautus* Laurent, 1943 and 51 species of gecarcinucid crabs; several diversifications within snake genera such as *Aspidura* Wagler, 1830 (Colubridae, 9 spp.), *Indotyphlops* Hedges et al., 2014 (Typhlopidae, 8 spp.) and *Rhinophis* Hemprich, 1820 (Uropeltidae, 17 spp.); as well as in lizard genera such as *Cnemaspis* Strauch, 1887 (Gekkonidae, 33 spp.), *Lankascincus* Greer, 1991 (Scincidae, 10 spp.), and *Ceratophora* Gray, 1835 (Agamidae, 6 spp.) (Bossuyt et al. 2004; Beenaerts et al. 2010; Agarwal et al. 2017; Meegaskumbura et al. 2019; Karunarathna et al. 2020, 2023; Wickramasinghe et al. 2022; Sampaio et al. 2023).

The island's freshwater fishes too, reflect this biogeographic pattern. About half of the ca. 100 species are associated with open habitats and a dry, seasonal climate. These occur ubiquitously in the lowland plains of both southern India and Sri Lanka and their colonization of the island dates to the Pleistocene (Pethiyagoda and Sudasinghe 2021). The rest of the island's endemic ichthyofauna is comprised mainly of small, endemic diversifications that are confined to the perhumid south-western quadrant and central hills. In some cases (e.g., the danionid genus *Devario* Heckel 1843, five species), multiple morphologically distinct species supported by substantial genetic divergences have been recognized (Sudasinghe et al. 2020a). In others, single, widely distributed species such as *Channa orientalis* Bloch & Schneider, 1801 (Channidae), *Lepidocephalichthys thermalis* (Valenciennes, 1846) (Cobitidae) and *Garra ceylonensis* Bleeker, 1863 (Cyprinidae), though morphologically conservative, are comprised of populations that are distinctly genetically divergent, representing multiple putatively cryptic species (Sudasinghe et al. 2020c, 2021b, 2023c).

Furthermore, previous studies have shown that despite the river basins that drain the island's perhumid south-western 'wet zone' traversing a shared coastal floodplain, their fishes exhibit remarkable genetic integrity, with limited evidence of geneflow between basins (Pethiyagoda and Sudasinghe 2021).

In this backdrop, we investigate the evolutionary relationships of the loach genus *Schistura* in Sri Lanka. With more than 200 putatively valid species distributed through South and Southeast Asia (Kottelat 2012), *Schistura* is by far the most speciose genus of Nemacheilidae. The genus is widely considered to be polyphyletic, however, serving as a catchall for diverse independent lineages of loaches (Sgouros et al. 2019; Dvořák et al. 2023). Three species are currently recognized within the genus in Sri Lanka: *Schistura notostigma* (Bleeker 1863), *S. madhavai* Sudasinghe, 2017 and *S. scripta* Sudasinghe, 2018. While the latter two are considered narrow-range endemics of the Walawe and Gin Rivers, respectively, the first is widely distributed across the central hills and south-western wet zone (Sudasinghe 2017, 2018). Described 'from Ceylon' by Bleeker (1863), the identity of *S. notostigma* has long been a matter of doubt because its type specimens were thought to be lost. These syntypes, however, were recently located among uncatalogued material in Naturalis Biodiversity Center, Leiden (RMNH), and examined by one of us (HS), allowing its tentative allocation to a natural population of *Schistura* (Sudasinghe et al. 2025).

Schistura notostigma is additionally interesting because it is among the few native freshwater fishes to have colonized Sri Lanka's central mountains and the Knuckles Massif to elevations above 1,200 m. Based on floristic and faunistic data, Pethiyagoda and Sudasinghe (2021) concluded that the ichthyofauna of these highlands suffered a recent climate-driven extinction event, probably in the Pleistocene. This evokes the hypothesis that the present highland populations of *S. notostigma* are recent colonists from the lowlands.

Here, through an examination of 261 preserved specimens available in museum collections, as well as molecular phylogenies constructed from nuclear and mitochondrial DNA sequences from 101 specimens representative of all wet zone river basins on the island, we investigate whether Sri Lankan *Schistura* are monophyletic, how many distinct evolutionary lineages they represent, what their inter- and intra-lineage relationships are, and what their phylogeographic structure is.

Based on this dataset, we also seek to test several hypotheses that emerge from previous work: (1) whether, like *Garra* Hamilton, 1822 (Sudasinghe et al. 2021b), *Schistura* too shows evidence of having undergone an extinction in the highlands; (2) whether populations of *Schistura* survived desiccation events, such as those known to have occurred in the Pleistocene, in rainforest refugia; (3) whether there is evidence of gene flow across watersheds; and (4) whether there is consonance between the morphological and genetic character-sets associated with the diversification of *Schistura* in Sri Lanka.

Material and methods

Study area

Sri Lanka is a 65,000 km^2 tropical continental island. Its south-west quadrant, the 'wet zone' (Fig. 1B), is characterized by a perhumid climate, with no consecutive months receiving an average rainfall below 100 mm. The remainder of the island has strongly seasonal rainfall, with an

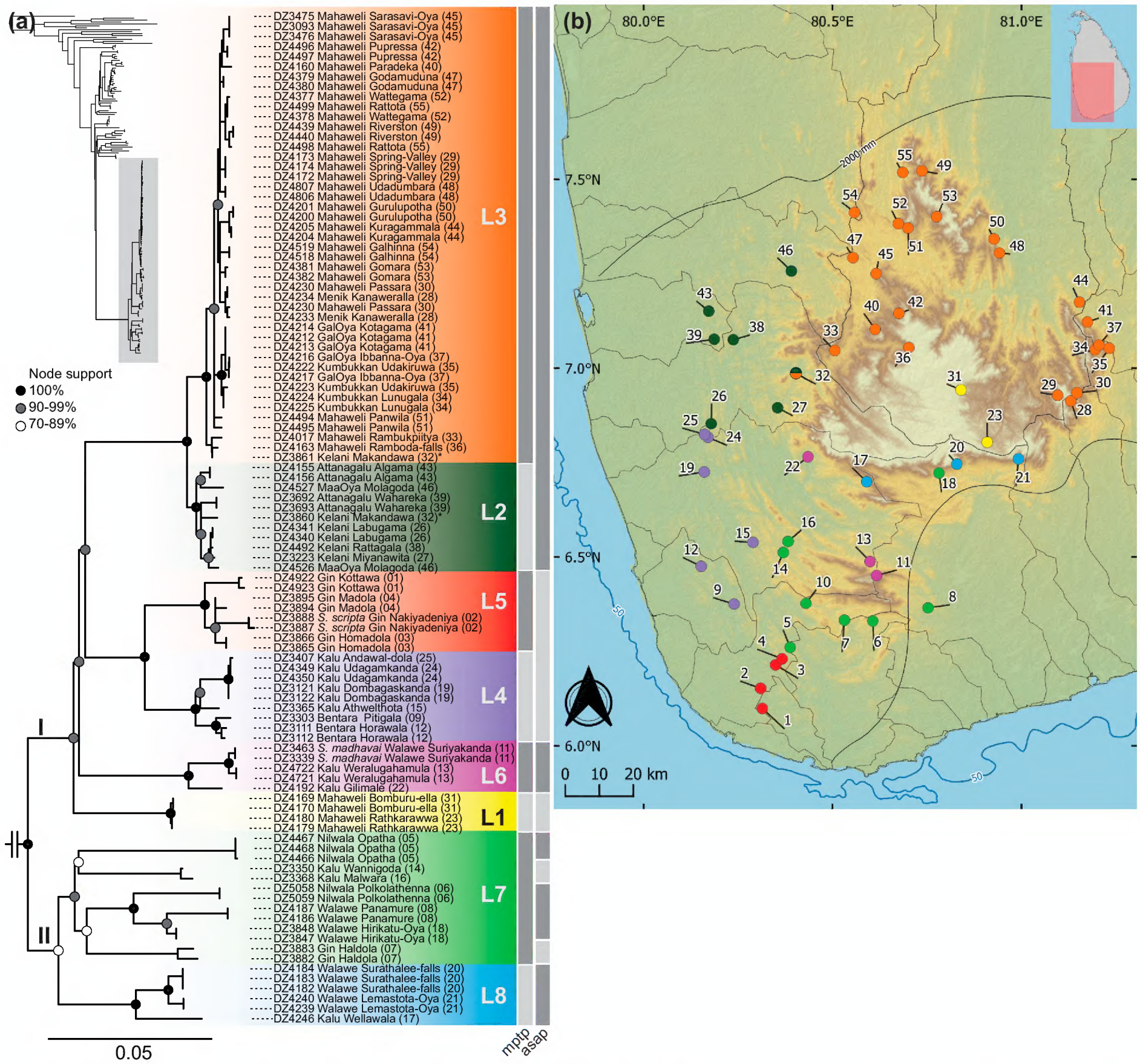

Figure 1. Molecular phylogenetic relationships of Sri Lankan *Schistura* based on the maximum likelihood inference of **A.** The concatenated mitochondrial DNA dataset (1,711 bp, 177 individuals). Node support represents ultrafast bootstrapping for 1,000 iterations. Node support below 70% is not labeled. Scale bar represents number of changes per site. The alternating light and dark-gray bars indicate results of the mPTP and ASAP molecular species delimitation analyses; **B.** Sampling localities for the molecular analysis. Major watersheds are marked with narrow black lines. Numbers on the phylogeny and the map represent sampling localities in Suppl. material 1.

extended dry period. The north-western region adjacent to the erstwhile Palk Isthmus, which connected the island to the Indian subcontinent during periods of low sea-levels, is especially arid and seasonal, with an average monthly rainfall below 50 mm for multiple periods of 3–4 months annually. The island's principal rivers descend from the central hills and the Knuckles Massif, which rise to 2,524 m above sea level (asl). Their western and southern slopes were once densely cloaked in rainforest, now anthropogenically reduced to less than 15% of their pre-colonial extent (Pethiyagoda and Sudasinghe 2021). The preponderance of the island's endemic biota is confined to these wet-zone forests, while the biota of the dry zone is mostly shared with South India (Pethiyagoda and Sudasinghe 2021). *Schistura* on the island are confined almost entirely to the wet and intermediate zones (rainfall > 2,000 mm/y) (Fig. 1B).

Fieldwork

Sampling for fish in Sri Lanka was carried out between 2014–2020 and followed ethical protocols approved by the University of Peradeniya. For the molecular analyses, we sampled 55 locations across the known range of *Schistura* in Sri Lanka (Fig. 1B, Suppl. material 1).

DNA protocols

Gene nomenclature follows the ZFIN Zebrafish Nomenclature Conventions (https://goo.gl/MdawKQ). We generated 101 *cox1* (GenBank PV926539–PV926639), 85 *cytb* (GenBank PV928017–PV928101), 59 *rag1* (GenBank PV928152–PV928210), and 50 *irbp* (GenBank

PV928102–PV928151) sequences (Suppl. material 1) from 102 samples collected at the 55 sites (Fig. 1B). The DNA extraction, PCR amplification, and product purification techniques followed those outlined by Sudasinghe et al. (2023a). We used ChromasPro v1.34 (Technelysium Pty Ltd, Australia) and MEGA v7.0 (Kumar et al. 2016) to verify and compile consensus sequences for the newly generated data.

We downloaded genetic data for *cox1*, *cytb*, *rag1*, and *irbp* for each representative of every nemacheilid lineage represented in the study of Dvořák et al. (2022). This included 75 nemacheilid lineages, together with *Cobitis taenia* Linnaeus 1758, which was used as the outgroup (Suppl. material 2). MUSCLE (Edgar 2004) in MEGA was used to align the sequences for *cytb* (1,084 bp), *cox1* (627 bp), *rag1* (1,470 bp), and *irbp* (714 bp). Alignments were examined and translated to ensure there were no frameshift mutations or premature stop codons. FASconCAT-G (Kück and Longo 2014) was used for data concatenation and sequence format conversion.

Phylogenetic analysis

Phylogenetic analysis using the Maximum Likelihood (ML) method was performed with IQ-TREE 2 (Minh et al. 2020) for each individual gene alignment: *cytb* (1,084 bp, 162 individuals), *cox1* (627 bp, 178 individuals), *rag1* (1,470 bp, 136 individuals), and *irbp* (714 bp, 127 individuals). The concatenated mitochondrial dataset (1,711 bp, 177 individuals) is referred to as the 'mtDNA dataset', while the concatenated nuclear dataset (2,184 bp, 149 individuals) is referred to as the 'nuDNA dataset'. ModelFinder (Kalyaanamoorthy et al. 2017) was used to determine the optimal nucleotide substitution model and dataset partitions, starting with each codon position treated as a separate subset. Node support in the ML tree was evaluated using ultrafast bootstrapping (BS) with 1,000 iterations (Hoang et al. 2018). IQ-TREE 2 analyses were carried out on UBELIX (http://www.id.unibe.ch/hpc), the HPC cluster at the University of Bern, Switzerland. The ML trees were visualized with Figtree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

To identify distinct molecular operational taxonomic units (MOTUs: Blaxter et al. 2005), we conducted single-gene species delimitation analyses for the *cox1* dataset. One approach employed tree-based molecular species delimitation using the Unix standalone software mptp 0.2.4 (Kapli et al. 2017). The "-multi" option in mptp was selected to implement the mPTP algorithm. A ML tree was reconstructed with IQ-TREE 2 for the *cox1* dataset that contains only the Sri Lankan *Schistura*, which served as the starting binary tree. Two analyses were performed with mPTP for the same dataset. The MCMC analysis was run for 50,000,000 generations, sampling every 10,000 generations, in two independent runs. The first 1,000,000 trees were discarded as burn-in, and convergence was verified by examining the plot of generation versus log-likelihood.

Additionally, species delimitation was performed using Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021), a distance-based method. ASAP was executed via its graphical web interface (https://bioinfo.mnhn.fr/abi/public/asap/#) for the Sri Lankan dataset of *Schistura* using default settings with p-distance, JC, and K80 models for the *cox1* dataset. The best dataset partition was identified based on the lowest ASAP score (Puillandre et al. 2021). In addition, uncorrected pairwise *cox1* genetic distances were calculated in MEGA.

To investigate phylogeographic patterns, haplotype networks for each mitochondrial marker were reconstructed using the Median Joining Network method (Bandelt et al. 1999) in PopArt (Leigh and Bryant 2015). Demographic histories were analyzed for each MOTU based on the *cytb* and *cox1* mitochondrial markers by calculating the number of haplotypes (h), polymorphic sites (S), parsimony-informative sites (P), nucleotide diversity (π), haplotype diversity (Hd), and performing neutrality tests, including Tajima's D (Tajima 1989) and Fu and Li's F (Fu and Li 1993), using DNAsp v.6 (Rozas et al. 2017).

Morphology

External morphology was examined in a total of 261 specimens representative of all MOTUs in Sri Lankan *Schistura*, based on the collections of the National Museum of Sri Lanka, Colombo (NH) and the Evolutionary Ecology and Systematics Lab, Department of Molecular Biology and Biotechnology, University of Peradeniya, Sri Lanka (DZ) (Suppl. material 3). The syntypes of *Nemacheilus notostigma* Bleeker, 1863 at RMNH too, were examined (Fig. 2).

Measurements were made point-to-point to the nearest 0.1 mm on the left side of specimens using digital calipers. This includes 29 linear measurements (Fig. 3, Suppl. material 4): Standard length (SL), Predorsal length (PDL), Postdorsal length (PoDL), Preanal length (PAL), Pre-anus length (PAnL), Prepelvic length (PPL), Caudal peduncle length (CPL), Caudal peduncle depth (CPD), Body depth (BD), Body width at dorsal-fin origin (BW), Dorsal-fin height (DFH), Dorsal-fin base length (DFBL), Anal-fin height (AFH), Anal-fin base length (AFBL), Pelvic-fin length (PFL), Pectoral-fin length (PeFL), Upper caudal lobe length (UCL), Lateral-line length (LL), LL absent length (LLA), Dorsal-head length (DHL), Lateral head length (LHL), Head depth at nape (HD), Head width at nape (HW), Snout length (SNL), Eye diameter (ED), Inter-orbital width (IOW), Maxillary barbel length (MaxB), Inner rostral-barbel length (InRosB), and Outer rostral-barbel length (OuRosB).

Additionally, 12 meristic counts were taken: lateral-line pores (LL-pores), branched dorsal-fin rays (DF), branched anal-fin rays (AF), branched pelvic-fin rays (PF), branched caudal-fin rays (CF), branched pectoral-fin rays (PeF), pre-dorsal bars, pre-dorsal interspaces, post-dorsal bars, post-dorsal interspaces, total bars, and total interspaces. The dark vertical bands on the body were counted as bars and the pale bands as interspaces. Any bar bifurcated on the side of the body was counted

Figure 2. Syntypes of *Schistura notostigma* (Bleeker, 1863), RMNH.PISC.2709. **A.** 54.0 mm SL; RMNH.PISC.2709; **B.** 54.8 mm SL; and RMNH.PISC.2709.C, 43.3 mm SL.

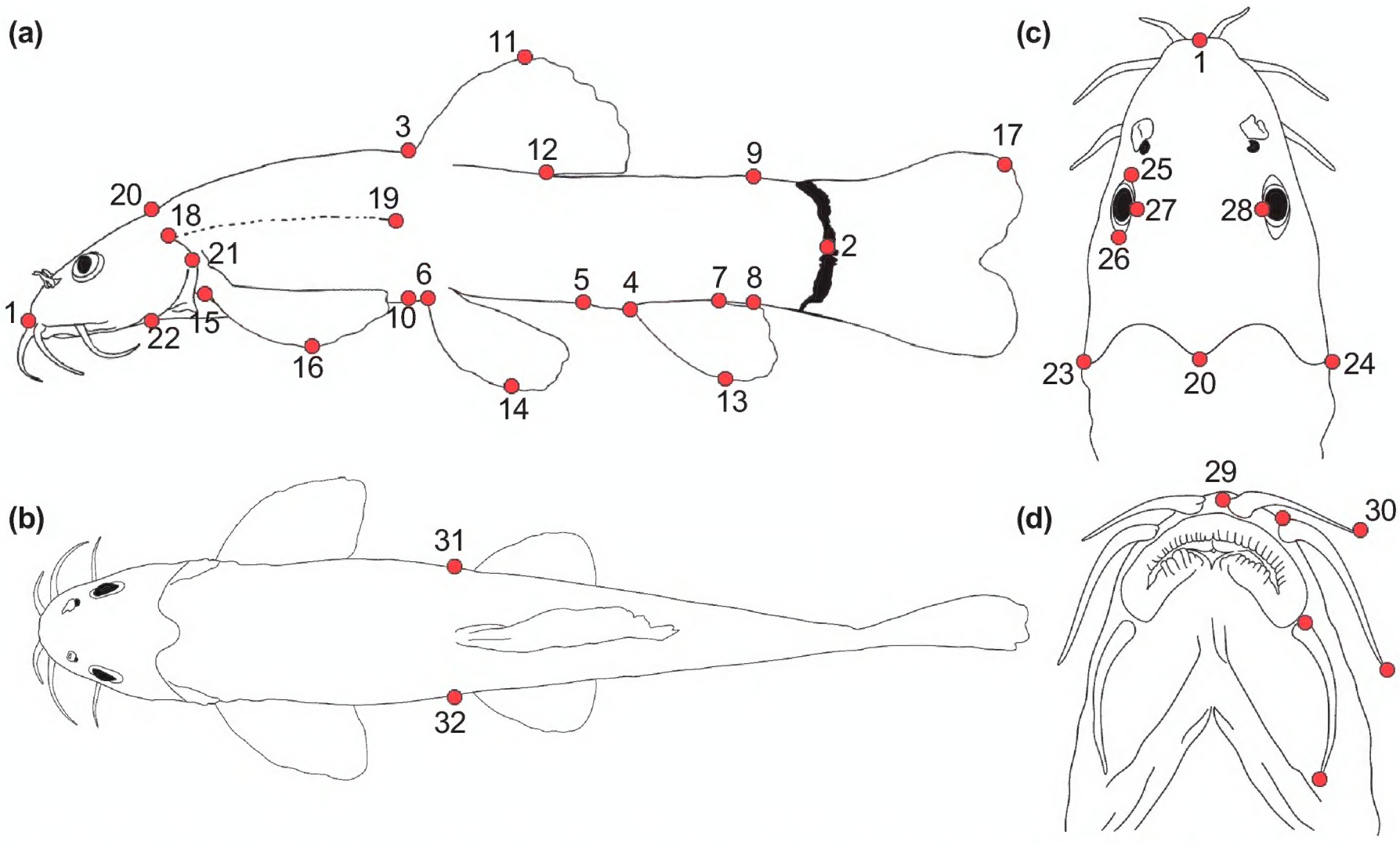

Figure 3. Landmarks for linear measurements taken from specimens of *Schistura*: **A.** Lateral; **B.** Dorsal; **C.** Dorsal head; **D.** Ventral head.

as two bars. Post-dorsal bars and post-dorsal interspaces refer to bars and interspaces posterior to the dorsal-fin origin. Among the meristic traits, CF did not show any variation and was excluded from further statistical analysis.

In addition, the following 15 categorical traits were recorded: lateral line position; shape of predorsal and postdorsal bars dorsally; width of predorsal and postdorsal bars; presence/absence of pelvic 'axillary' lobe; position of anus; pigmentation on upper and lower lips; dorsal-fin base color pattern; color pattern of dorsal-fin rays; pectoral-fin color pattern; shape of caudal-fin bar; and position of adipose layer. The details of character coding for these traits are provided in Suppl. material 4.

Statistical analyses

R Statistical Software 4.0.0 (R Core Team 2020) was used for all statistical analyses. All measurements and meristic traits were log-transformed prior to analysis. Using standard length (SL) as the size axis, the remaining 39 traits were plotted against SL to verify positive correlations and identify potential outliers. All linear measurements showed a positive correlation with SL: no outliers were detected.

Principal Component Analyses (PCA) were conducted to visualize and summarize multivariate morphometric data. Morphometric analyses were used to determine whether the MOTU of Sri Lankan *Schistura* derived from the molecular analyses formed distinct clusters in morpho-space. The dataset was size-corrected following the methods of Berner (2011) and Roesti et al. (2023) by performing a pooled within-group regression, with MOTU identity as the 'group' during size correction. In this approach, an ANCOVA was performed for each MOTU and trait, treating the log-transformed trait as the response variable, MOTU as a factor, and log-transformed size as a covariate. Size-corrected trait values were obtained by adding the residuals to the predicted trait value for each MOTU at the mean size across all individuals. PCA was then performed using a correlation matrix to explore and summarize the multivariate morphometric and meristic data in multiple dimensions, employing the R package FactoMineR (version 1.34) (Lê et al. 2008). To evaluate whether the PCA components differed significantly among MOTUs, an ANCOVA was conducted using the lm() function with the model syntax lm(PC ~ MOTU) in R. Type III sums of squares, calculated with the car package (Fox and Weisberg 2018), were used to determine F-statistics. Additionally, a multiple correspondence analysis (MCA) was carried out using FactoMineR to summarize 15 categorical characters and assess whether MOTUs formed distinct clusters. Furthermore, for these categorical traits, a Fisher's Exact Test was carried out to assess whether each trait was significantly different among the various MOTUs of Sri Lankan *Schistura*.

A one-way ANOVA using the aov() function in R was conducted to evaluate body-size differences among the MOTUs. Here, standard length (SL) served as a proxy for body size. Post-hoc comparisons were performed using the Tukey HSD method in base R to identify specific size differences between the MOTUs. Additionally, to identify morphometric and meristic traits that differed significantly between MOTUs, an ANCOVA was applied to each log-transformed trait using the model lm(trait ~ MOTU + SL), with SL as a covariate. Field observations indicated that *Schistura* specimens collected at higher elevations tend to grow larger and that larger specimens tend to exhibit fewer body bars. We tested whether there is in fact a significant positive relationship between elevation and body size, and a significant negative relationship between the number of bars and body size, by using a Pearson correlation test.

Results

Mitochondrial phylogeny

The mitochondrial DNA (mtDNA) phylogenetic analysis (Fig. 1A) robustly supports the monophyly of Sri Lankan *Schistura* (bootstrap support [BS] = 100). The mPTP species delimitation analysis identified eight distinct lineages, or molecular operational taxonomic units (MOTUs), which are designated L1–L8 (Fig. 1A). These lineages are divided into two primary clades: Clade I, comprising six lineages (L1–L6), and Clade II, containing two (L7 and L8).

The delineation of these eight MOTUs reveals a degree of phylogeographic structuring, with a few notable exceptions. Within the nominal species, the topotypes of *Schistura scripta* and *Schistura madhavai* correspond to L5 and L6, respectively. Several specimens from the Gin Basin (Kottawa, Madola, and Homadola), located near Nakiyadeniya, the type locality of *S. scripta*, cluster with L5. Similarly, specimens from Weralugahamula and Gilimale in the headwaters of the Kalu Basin cluster with *S. madhavai*.

In the Mahaweli Basin (which, because of its size, was the most extensively sampled region in this study), two genetically distinct lineages were identified. These do not have a sister-group relationship. L1 is a narrow-range lineage restricted to samples from downstream Bomburu-ella and Rathkarawwa at elevations between 1,155 and 1,182 m asl. It is the sister group of the remaining lineages within Clade I (BS = 99). The other lineage, L3, encompasses samples from 17 localities across the Mahaweli Basin, spanning the elevation range 243–1,266 m asl. All specimens from the headwaters of the east-flowing Menik, Kumbukkan, and Gal river basins, cluster with L3.

Samples from the headwaters of the Maa, Attanagalu, and Kelani rivers (84–497 m asl) are represented by L2, which has a sister-group relationship to L3 (BS = 100). Within the Kelani Basin, two samples from Makandawa (locality 32), in close proximity to the headwaters of the Mahaweli watershed, have unique affiliations: DZ3860 clusters with L2, while DZ3861 clusters with L3.

L4 occurs in the lowlands (25–157 m asl) of the Kalu and Bentara basins in the southwestern wet zone. It has a sister-group relationship to L5 (BS = 100).

Clade II includes L7 and L8. These represent mid-elevation (125–648 m asl) and higher elevation (571–835 m asl)

lineages, respectively. L7 encompasses samples from the Kalu, Gin, Nilwala, and Walawe basins, forming a sister group relationship with L8 (BS = 85). Several subclades are evident within L7, while L8 represents specimens from the Kalu and Walawe basin headwaters.

The ASAP species delimitation analysis corroborates the distinctiveness of L1, L6, and L8 while dividing L7 into four sublineages. Furthermore, the ASAP analysis clusters L2 and L3 as a single lineage, as it does also L4 and L5. The patterns of intra- and inter-lineage genetic divergence among these lineages are summarized in Fig. 4, Suppl. material 5. The highest intra-lineage divergence is observed in L7 (0–6.4%), while all other lineages exhibit divergence values in the range of 0–3.5% (Fig. 4, Suppl. material 5). Inter-lineage divergence between MOTUs exceeds 4% except between L2 and L3 (1.1–2.9%) and L4 and L5 (3.1–4.3%).

Nuclear phylogeny

The nuclear DNA (nuDNA) phylogeny (Fig. 5) strongly supports the monophyly of Sri Lankan *Schistura* (bootstrap support [BS] = 100). However, the relationships among the various lineages are poorly resolved, with the eight mitochondrial DNA (mtDNA) lineages failing to form monophyletic groups and instead presenting as a polytomy (Fig. 5).

Genetic diversity and phylogeography

The genetic diversity metrics for each mitochondrial marker—including the number of haplotypes, polymorphic sites, parsimony-informative sites, nucleotide diversity, haplotype diversity, and neutrality test outcomes—are presented in Suppl. material 6. Neutrality tests (Tajima's D and Fu and Li's F) were negative and statistically significant for both *cytb* and *cox1* in lineage L3, the most widespread of the highland lineages. None of the other MOTUs exhibited significant results in either neutrality test. The highest nucleotide diversity was observed in L7 (for both *cytb* and *cox1*), while haplotype diversity values were comparable across most MOTUs (Suppl. material 6).

L1 is represented by only two haplotypes in the *cytb* and *cox1* haplotype networks (Fig. 6). Among the MOTUs, L2 (Fig. 7), L3 (Fig. 8), L4 (Fig. 9), L5 (Fig. 10), L6 (Fig. 11), L7 (Fig. 12), and L8 (Fig. 13) all include haplotypes from multiple contiguous river basins, except for L5, which contains haplotypes exclusively from the Gin River basin. Haplotype sharing between river basins, however, is rare and observed only in L3.

In the *cytb* haplotype network of L3, haplotype H20 is shared between the Mahaweli and Menik basins, while H24 is shared between the Gal and Kumbukkan basins. Similarly,

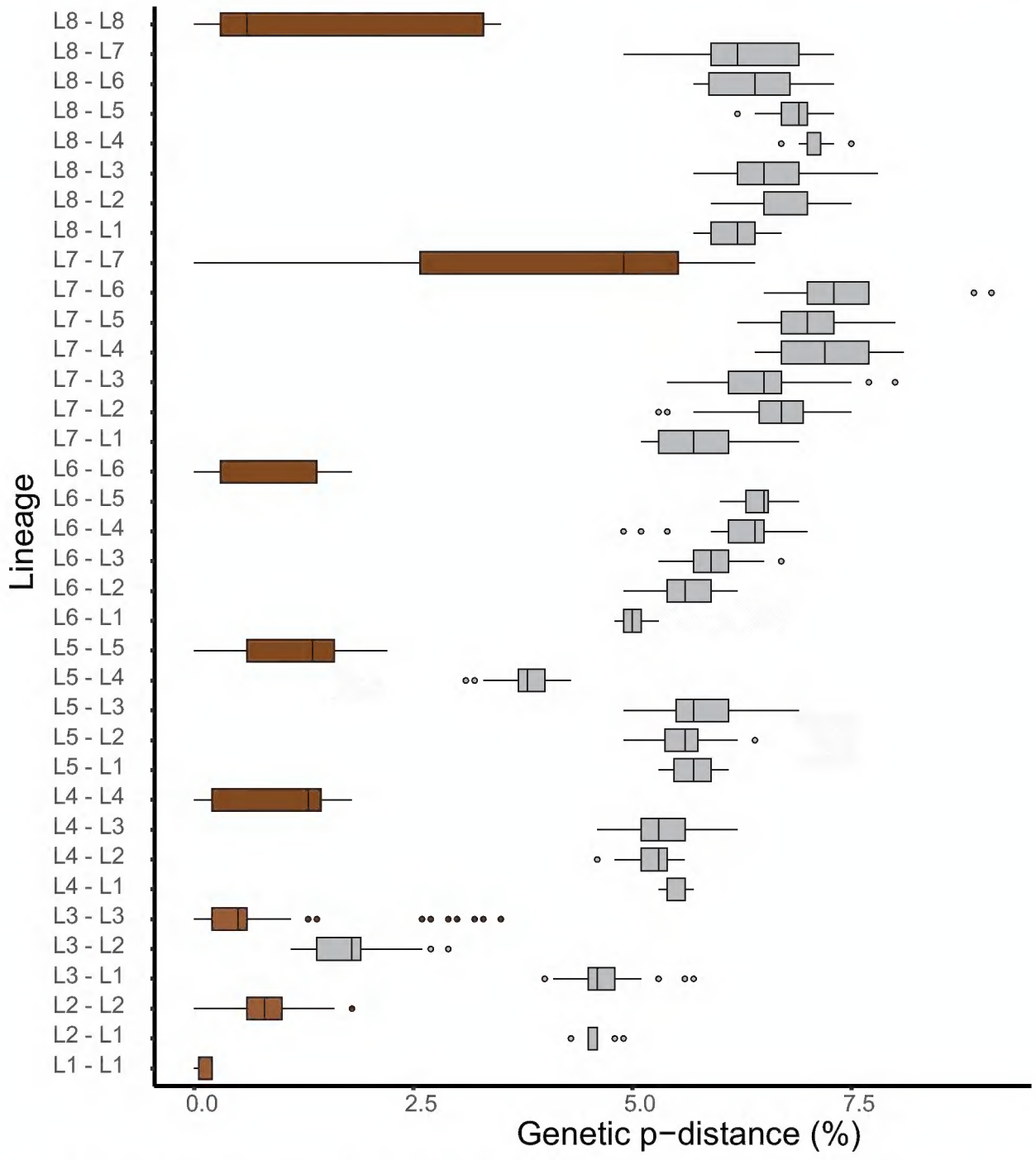

Figure 4. Variation of intra-lineage (brown) and inter-lineage (gray) genetic variation in the mitochondrial cox1 molecular marker for Sri Lankan *Schistura*.

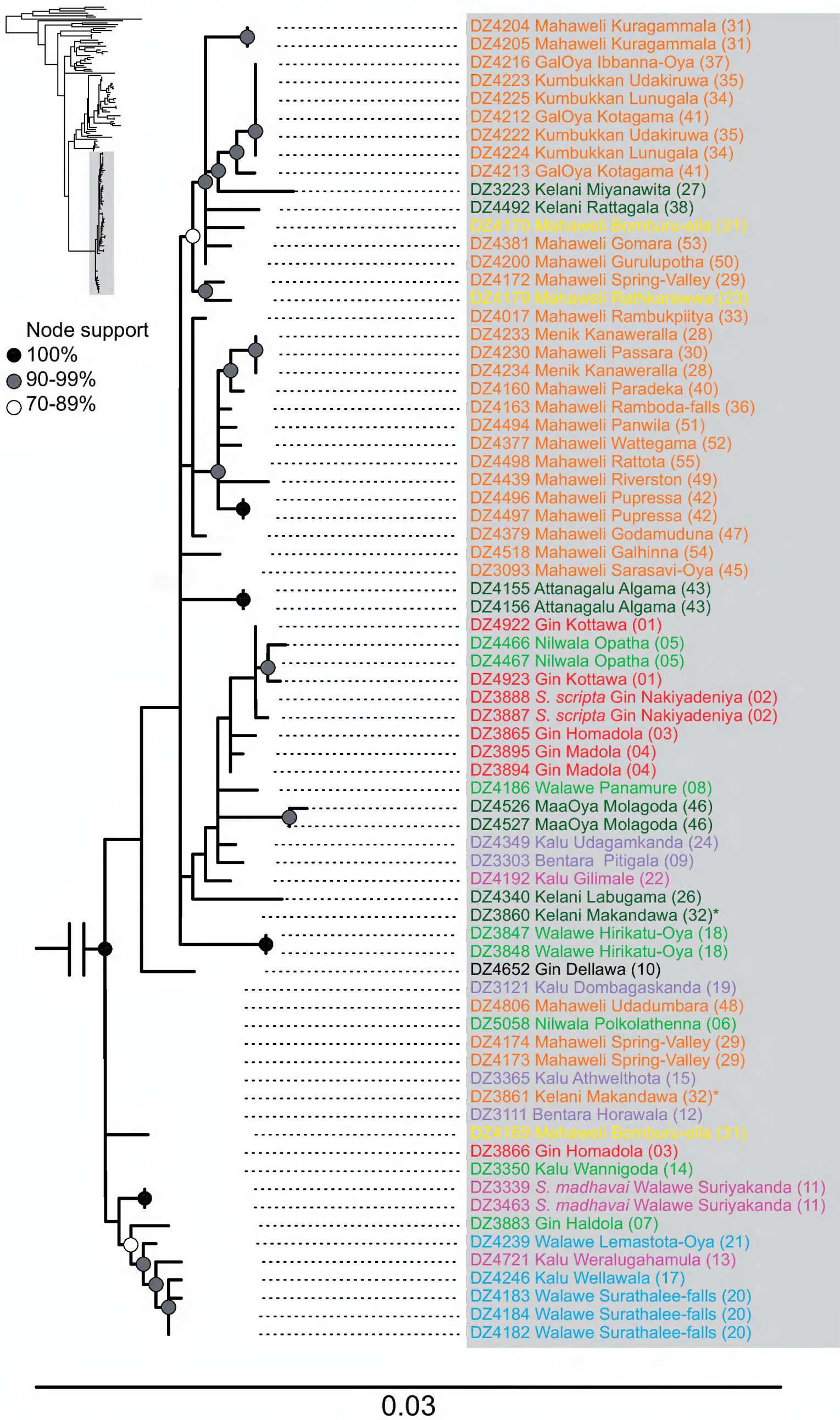

Figure 5. Molecular phylogenetic relationships of Sri Lankan *Schistura* based on maximum likelihood inference of the concatenated nuclear DNA dataset (2,184 bp, 149 individuals). Node support represents ultrafast bootstrapping for 1,000 iterations. Node support below 70% is not labeled. Scale bar represents the number of changes per site. Numbers on the phylogeny represent the sampling localities in Suppl. material 1. Color codes indicate the MOTUs recovered in the mitochondrial phylogeny (Fig. 1).

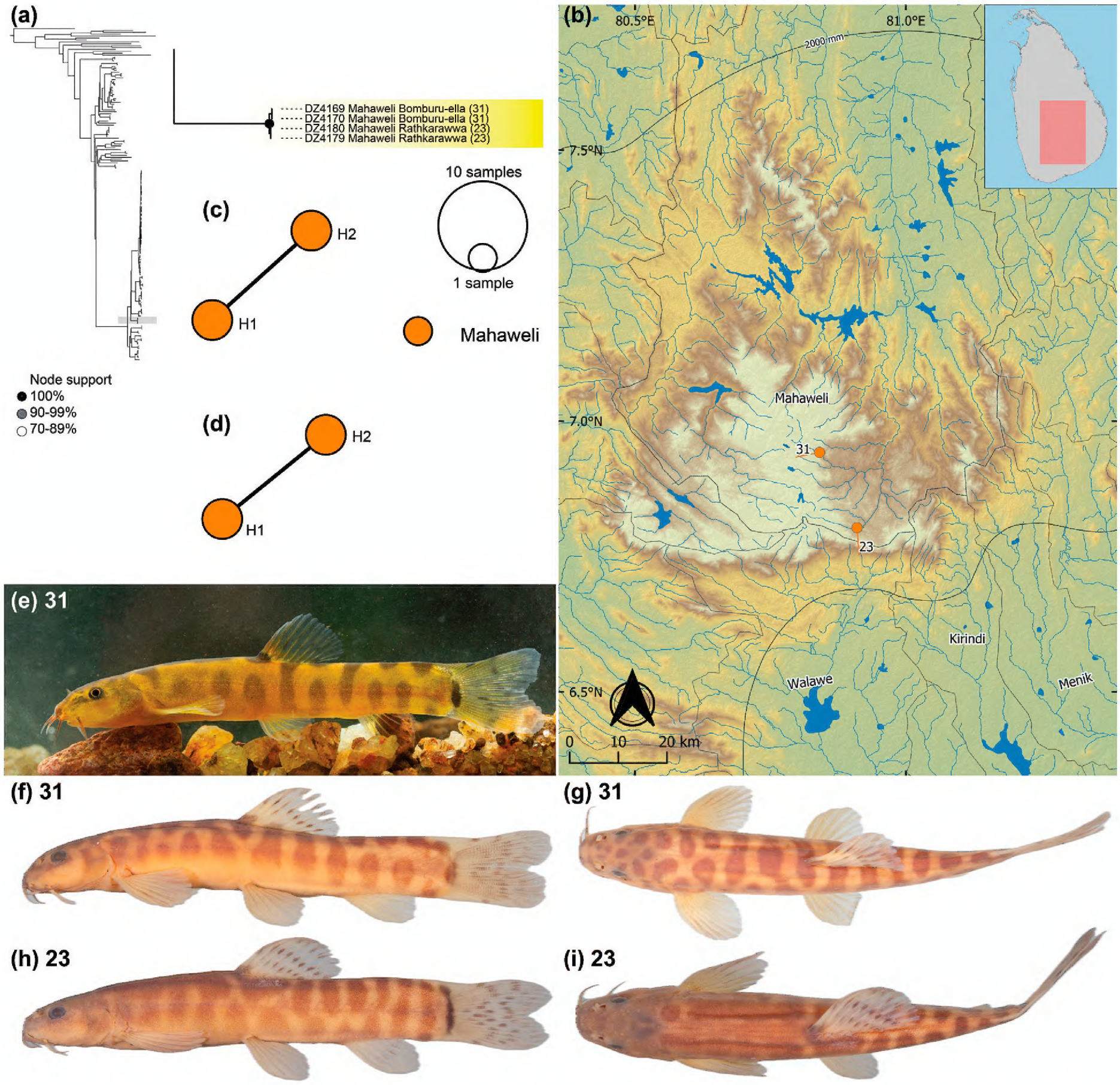

Figure 6. Lineage1: **A.** Phylogenetic position; **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E.** Live coloration pattern of lineage 1; **F, H.** Lateral, and **G, I.** Dorsal view of specimen coloration in lineage 1. Numbers represent the sampling localities listed in Suppl. material 1.

in the *cox1* haplotype network of L3, haplotypes H17 and H15 are shared between the Mahaweli and Menik basins and the Mahaweli and Kelani basins, respectively. Furthermore, H16, the most frequent haplotype in L3, is shared across the Mahaweli, Gal, and Kumbukkan basins (Fig. 8).

Morphological analysis

The PCA of log-transformed, size-corrected morphometric and meristic data reveals PC1 and PC2 collectively account for 29.66% of the total variance (Fig. 14A, Suppl. material 7). The PC1 vs. PC2 plot identifies distinct clusters for lineages L1, L3, L5, and L8, indicating inter-lineage morphological divergence (Fig. 14A). In contrast, lineages L2, L4, L6, and L7 mostly overlap in morphological space. The primary axis of morphological variation, PC1, explains 17.07% of the total variation (Fig. 14A: 'MOTU', $F = 45.32$, $P < 0.0001$), while PC2 accounts for 12.59% (Fig. 14: 'MOTU', $F = 21.08$, $P < 0.0001$).

Along the PC1 axis, lineages L3 and L8 exhibit higher average trait values for heavily loaded variables such as pectoral fin length, upper caudal-lobe length, lateral head length, head width at nape, and dorsal-fin base length (Suppl. material 7). PC2, on the other

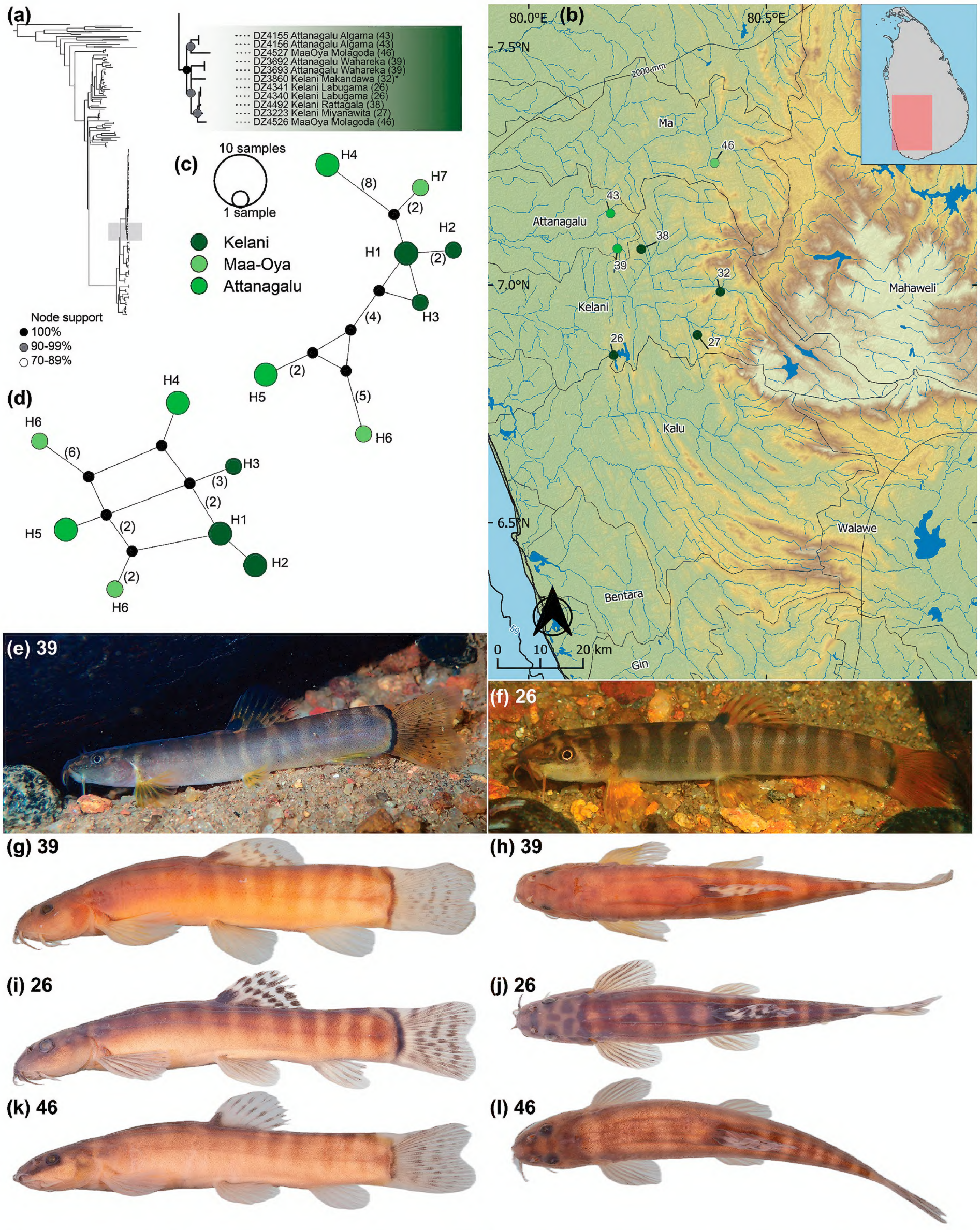

Figure 7. Lineage 2: **A.** Phylogenetic position; **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E, F.** Live coloration pattern of lineage 2; **G, I, K.** Lateral, and **H, J, L.** Dorsal view of specimen coloration in lineage 2. Numbers represent the sampling localities listed in Suppl. material 1.

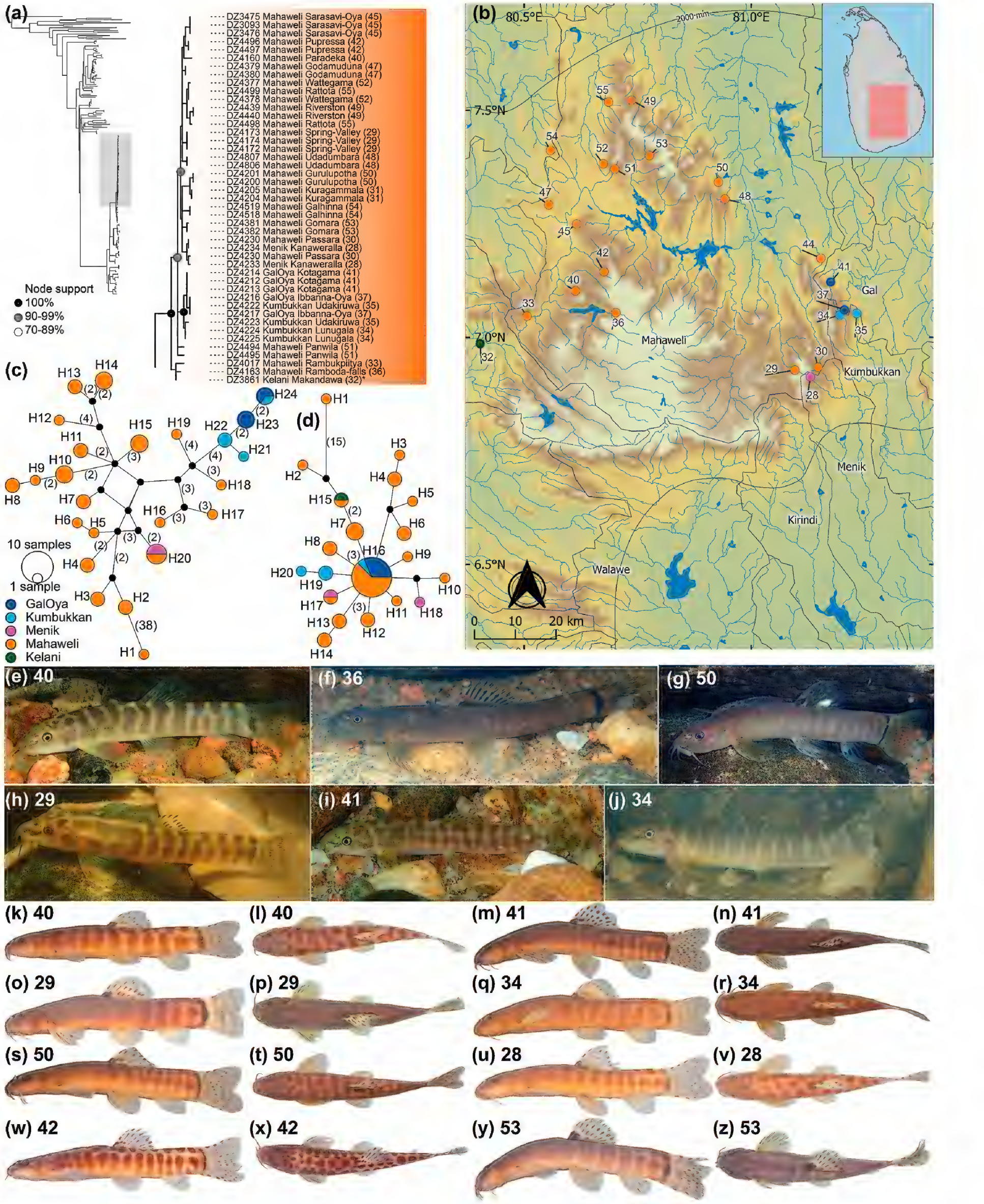

Figure 8. Lineage 3: **A.** Phylogenetic position; **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E–J.** Live coloration pattern of lineage 3; **K, M, O, Q, S, U, W, Y.** Lateral, and **L, N, P, R, T, V, X, Z.** Dorsal view of specimen coloration in lineage 3. Numbers represent the sampling localities listed in Suppl. material 1.

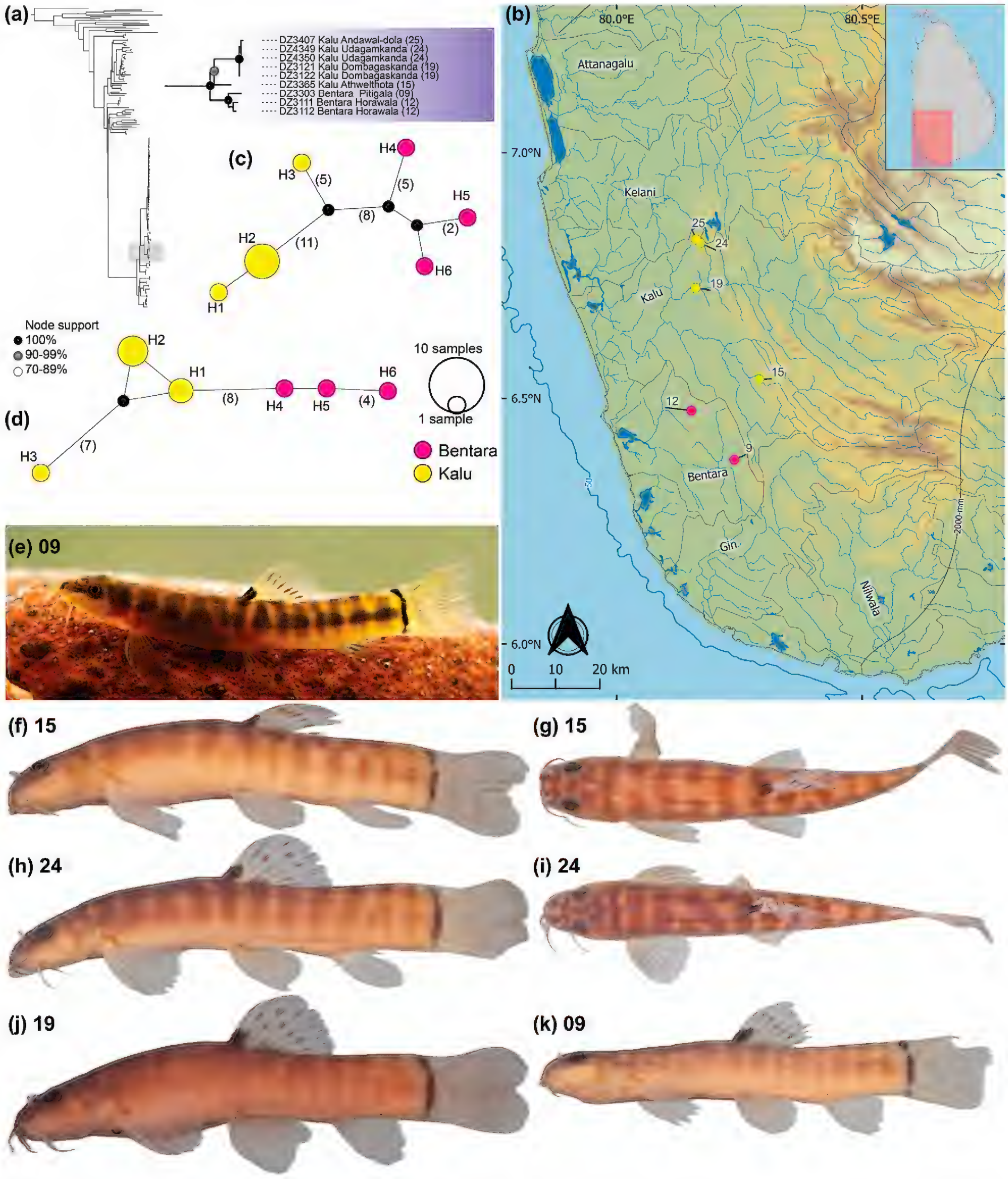

Figure 9. Lineage 4: **A.** Phylogenetic position; and **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E.** Live coloration pattern of lineage 4; **F, H, J.** Lateral, and **G, I, K.** Dorsal view of specimen coloration in lineage 4. Numbers represent the sampling localities listed in Suppl. material 1.

hand, differentiates L2, L4, L6, L7, and L8, which exhibit larger average values compared to L1, L3, and L5 (Fig. 14A). The most heavily loaded traits on PC2 include the number of body bars and interspaces, reflecting variations in color pattern (Suppl. material 7).

The MCA of categorical traits reveals that the first and second dimensions account for 17.4% of the total variance (Fig. 14B). Dimension 1 is primarily explained by traits such as the position of the anus, extent of body scalation, and shape of the caudal-fin bar, while Dimension 2 is influenced

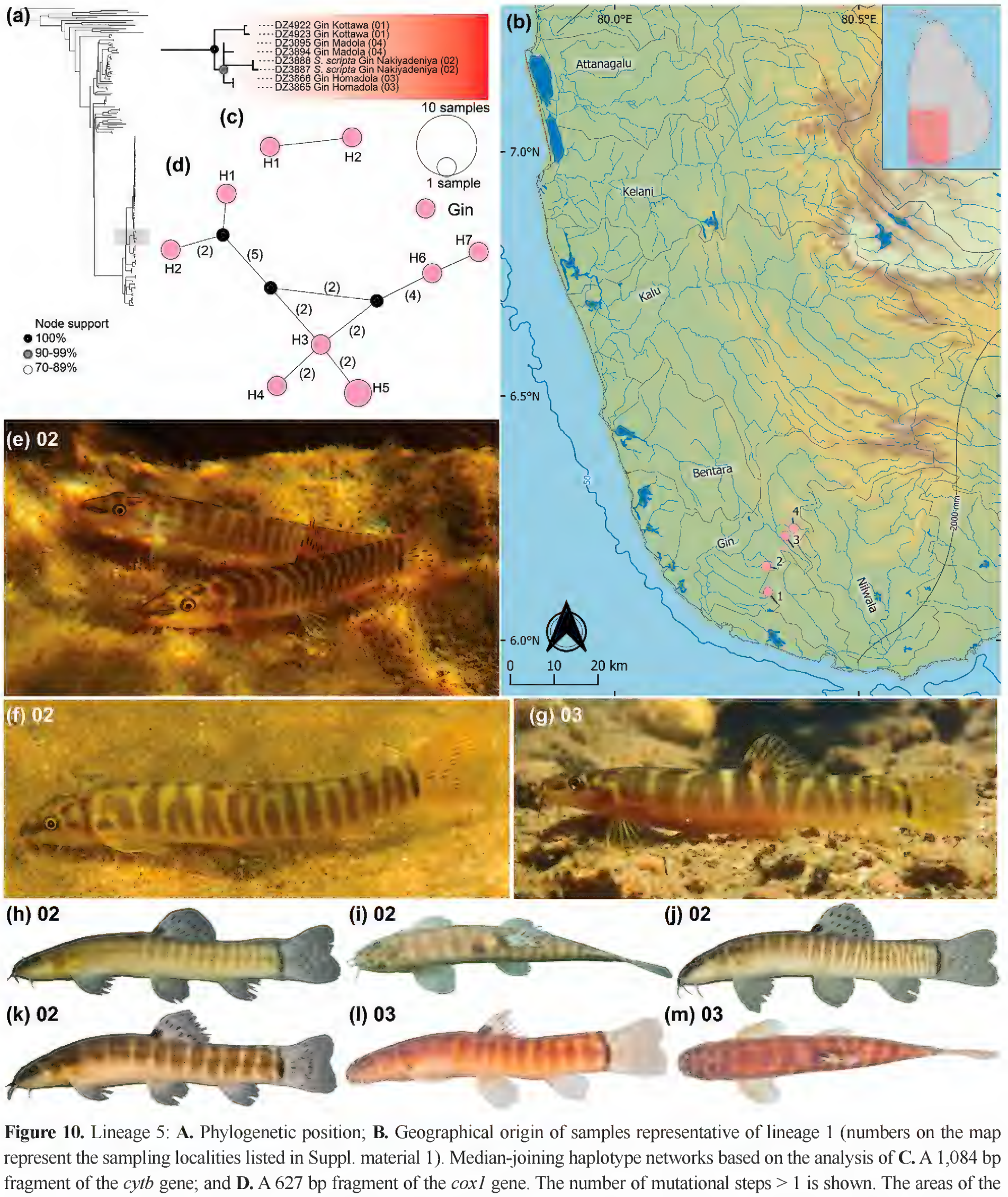

Figure 10. Lineage 5: **A.** Phylogenetic position; **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E–G.** Live coloration pattern of lineage 5; **H, J, K, L.** Lateral, and **I, M.** Dorsal view of specimen coloration in lineage 5. Numbers represent the sampling localities listed in Suppl. material 1.

by traits such as the width of pre- and post-dorsal bars and the dorsad shape of predorsal bars. The MOTUs show significant variation along both Dimension 1 ($F = 44.02$, $P < 0.0001$) and Dimension 2 ($F = 25.98$, $P < 0.0001$) (Fig. 14B).

Suppl. material 8 summarizes the morphometric and meristic data for all examined specimens. A one-way ANOVA shows significant differences in log-transformed standard length across MOTUs ($F = 12.14$, $P = 2.413e\text{-}14$; Fig. 15A). Post-hoc Tukey HSD tests reveal significant size differences between multiple MOTU pairs, with the largest differences observed between L3 and several other MOTUs (e.g., L4, L5, and L7; $P < 0.001$).

The ANCOVA results indicate significant interspecific variation in most morphometric and meristic traits,

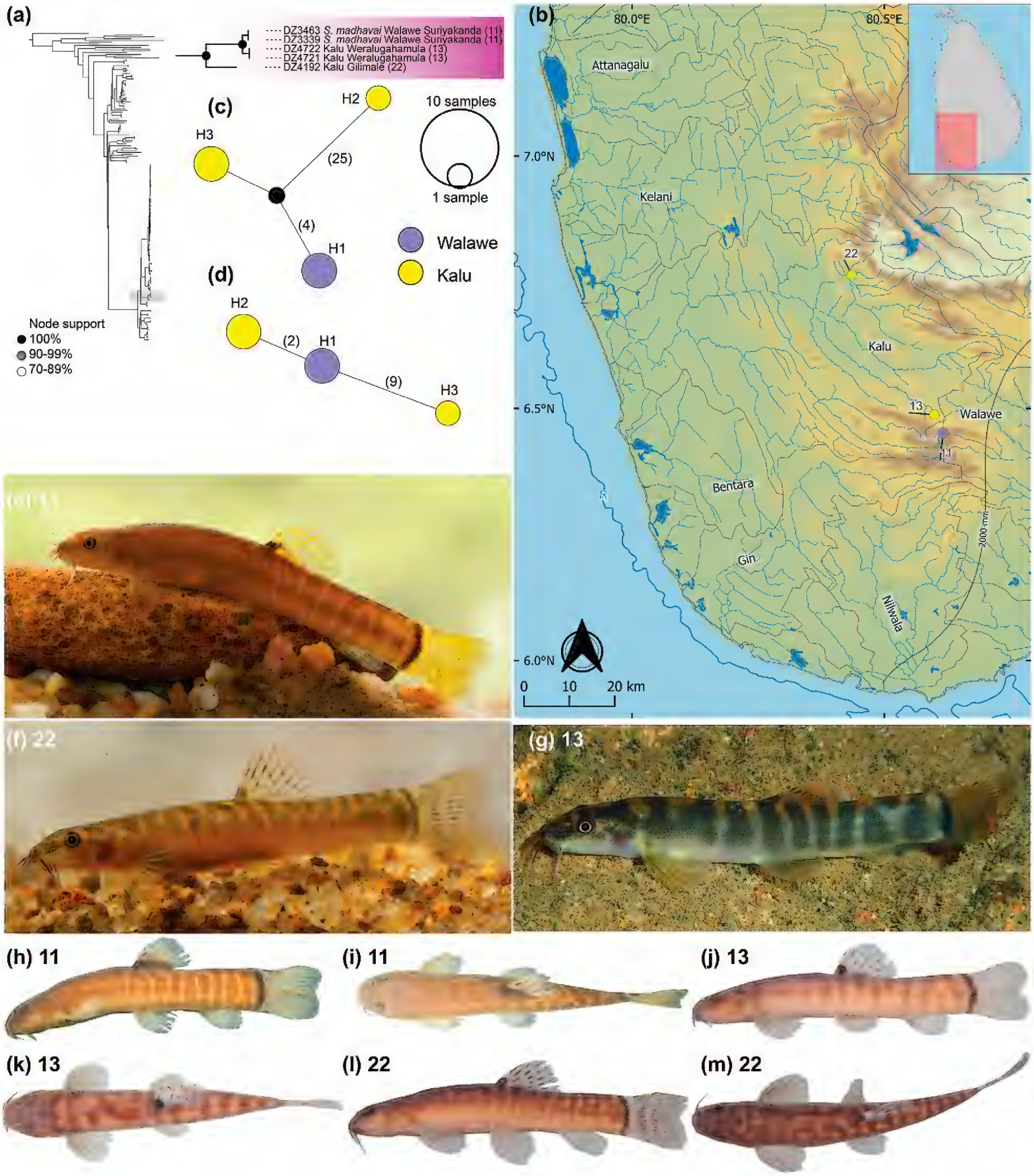

Figure 11. Lineage 6: **A.** Phylogenetic position; **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E–G.** Live coloration pattern of lineage 4; **H, J, L.** Lateral, and **I, K, M.** Dorsal view of specimen coloration in lineage 6. Numbers represent the sampling localities listed in Suppl. material 1.

exceptions being the inner rostral barbel length, outer rostral barbel length, and number of branched anal-fin rays (Suppl. material 8). The total number of body bars varies among MOTUs, with L5 exhibiting the highest average number and L2 and L8 the lowest (Fig. 15B).

Additionally, there is a significant positive correlation between elevation and standard length (r = 0.473, t(259) = 8.646, *P* = 5.629e-16; Fig. 15D) and a significant negative correlation between the number of body bars and standard length (r = -0.3414, t(259) = -5.847, *P* = 1.5e-08; Fig. 15C).

The proportion of specimens with 7½ versus 8½ branched dorsal-fin rays varies by lineage. Lineages L4 (N = 11), L5 (16), L6 (11), and L7 (22) predominantly

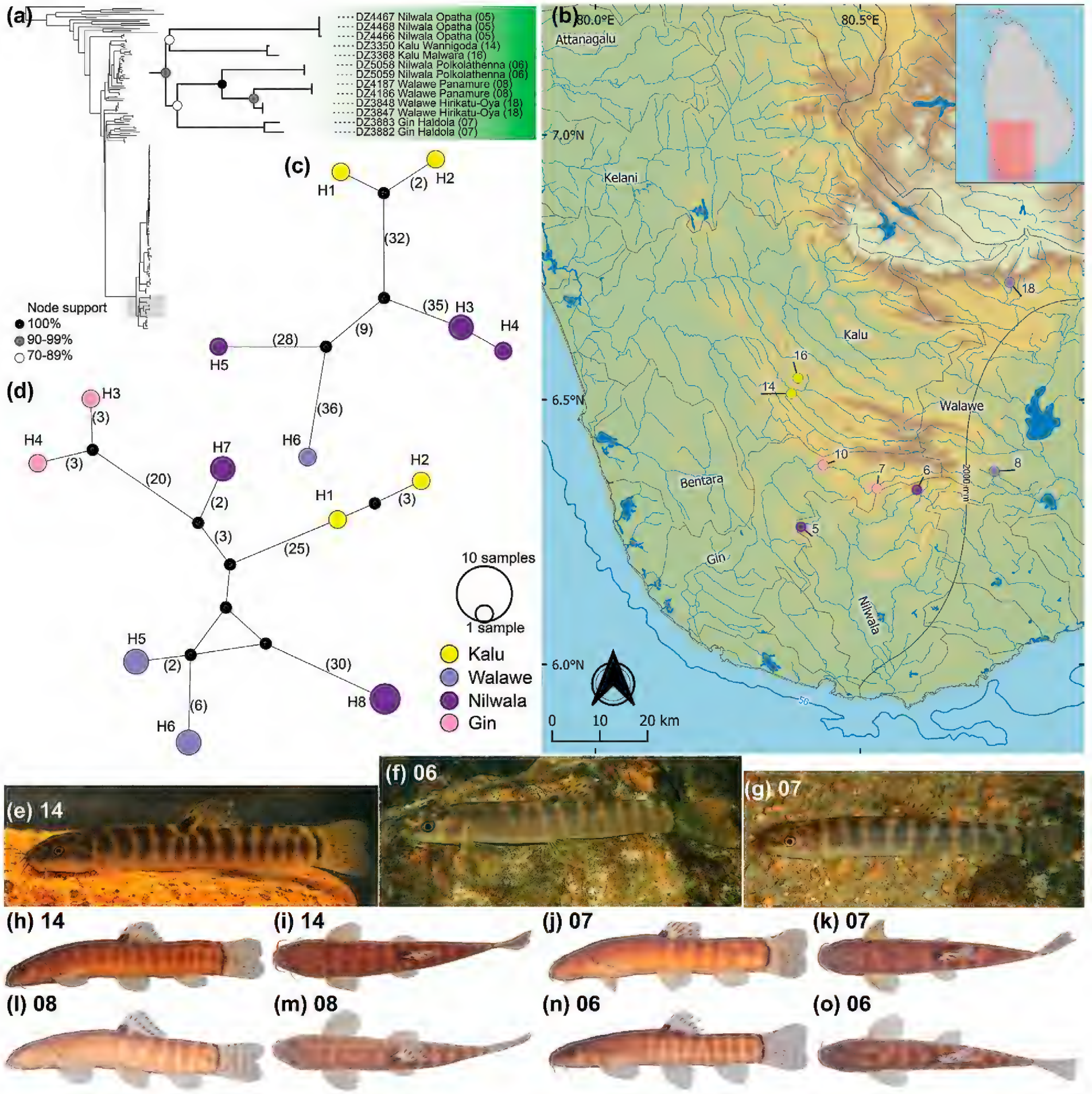

Figure 12. Lineage 7: **A.** Phylogenetic position; **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E–G.** Live coloration pattern of lineage 7; **H, J, L, N.** Lateral, and **I, K, M, O.** Dorsal view of specimen coloration in lineage 7. Numbers represent the sampling localities listed in Suppl. material 1.

exhibit 7½ branched rays (>75% of specimens), while L1 (7), L3 (130), and L8 (29) primarily exhibit 8½ branched rays (>75% of specimens) (Fig. 16). In L2 (35), however, 65% of specimens possess 8½ branched rays. Interestingly, lineages with mostly 8½ branched rays (L1, L3, and L8) are associated with the highlands, whereas those with mostly 7½ are from the lowlands.

Fisher's exact test results for 15 categorical traits are summarized in Suppl. material 9. L1 is distinguished by a wide, incomplete caudal-fin bar (Fig. 17D), while L8 and L6 are characterized by a wide, complete bar (Fig. 17B) in more than 80% and 65% of specimens, respectively. The remaining MOTUs predominantly exhibit a narrow but complete caudal-fin bar (Fig. 17C).

The frequency of the pelvic axillary lobe too, exhibits an altitudinal distribution (Fig. 18): more than 50% of specimens in highland lineages (L1, L3, and L8) possess a rudimentary or distinct pelvic axillary lobe (Fig. 18C, D), a character absent in most specimens of from lowland lineages (L2, L4, L5, L6, and L7) (Fig. 18B). Similarly, highland lineages exhibit distinct body scales only to just beyond the dorsal-fin origin, whereas in L5, body scales

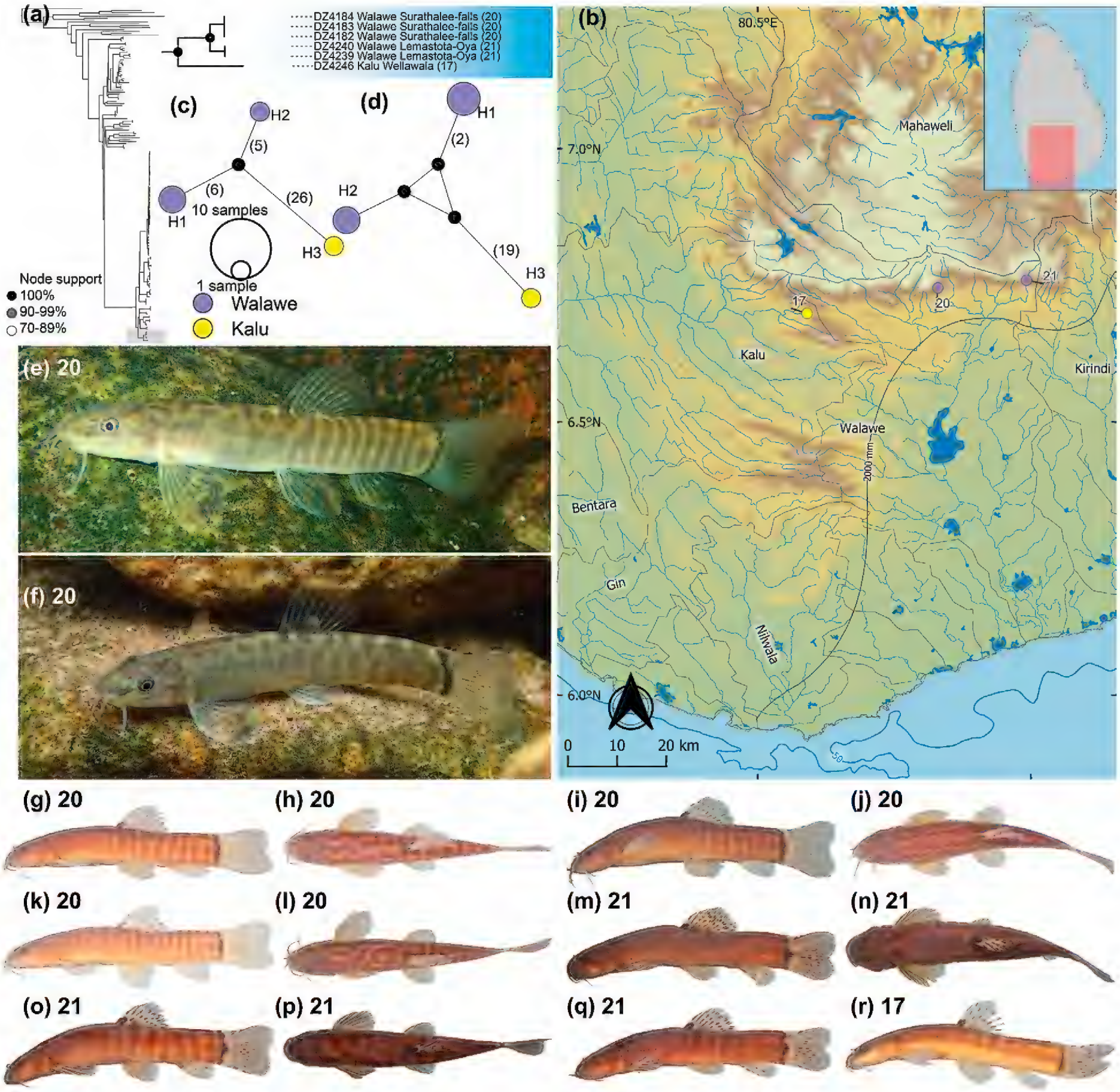

Figure 13. Lineage 8: **A.** Phylogenetic position; **B.** Geographical origin of samples representative of lineage 1 (numbers on the map represent the sampling localities listed in Suppl. material 1). Median-joining haplotype networks based on the analysis of **C.** A 1,084 bp fragment of the *cytb* gene; and **D.** A 627 bp fragment of the *cox1* gene. The number of mutational steps > 1 is shown. The areas of the circles are proportional to the number of individuals sharing a given haplotype. Black circles indicate hypothetical nodes. Legend colors correspond to river basins. Major watersheds are marked in narrow black lines; **E, F.** Live coloration pattern of lineage 8; **G, I, K, M, O, Q, R.** Lateral, and **H, J, L, N, P.** Dorsal view of specimen coloration in lineage 8. Numbers represent the sampling localities listed in Suppl. material 1.

are distinct throughout (Fig. 19). Lineages L2, L4, L6, and L7 each show an inconsistent distribution of body scales (Fig. 19A).

The number of lateral-line pores too, varies considerably across MOTUs. L5 has the highest average number of lateral-line pores, while L7 and L8 have the lowest (Fig. 20A). In most specimens of L1, L3, and L5, the lateral line extends to between the dorsal- and anal-fin origins, while in L7, it ends anterior to the dorsal-fin origin (Fig. 20B). L2 and L6 exhibit intermediate conditions, with the lateral line extending beyond the dorsal-fin origin in 50% and 65% of specimens, respectively.

Discussion

Phylogeny

Although twelve putatively valid species of *Schistura* have been reported from the peninsula of India (*S. altipedunculatus* (Bănărescu & Nalbant, 1968), *S. bhimachari* (Hora, 1937), *S. denisoni* (Day, 1867), *S. hiranyakeshi* Praveenraj et al., 2020, *S. kodaguensis* (Menon, 1987), *S. mukambbikaensis* (Menon, 1987), *S. nagodiensis* Sreekantha et al., 2006, *S. nilgiriensis* (Menon, 1987), *S. pambaensis* (Menon, 1987), *S. rendahli* (Bănărescu &

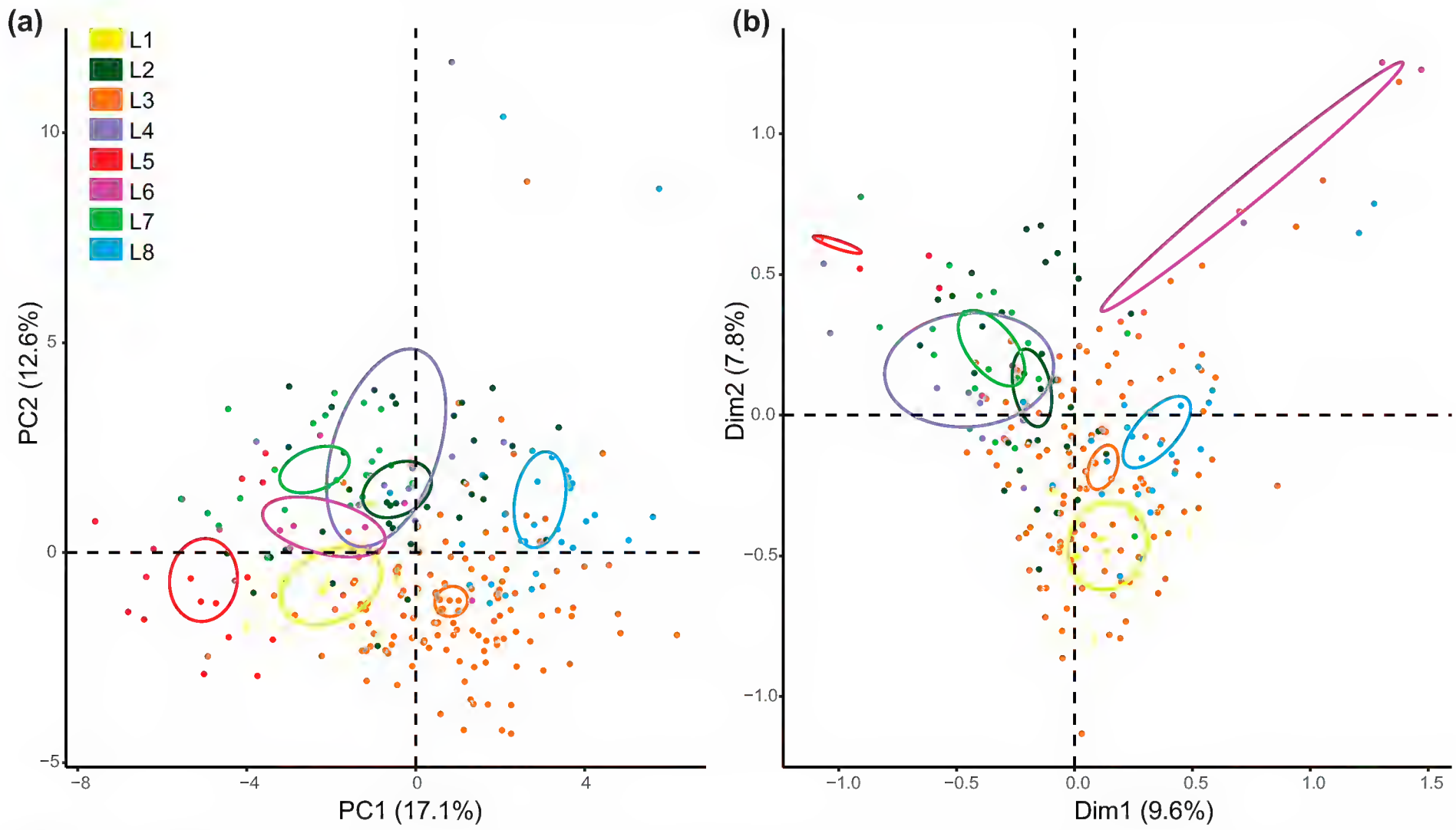

Figure 14. A. PCA biplot of the first two principal components of morphological variation (size-corrected) in 39 counts and measurements of Sri Lankan *Schistura* (n = 261); **B.** MCA biplot of the first two dimensions of 15 categorical traits (n = 261). Ellipses represent 95% confidence intervals around group mean points.

Nalbant, 1968), *S. semiarmatus* (Day, 1867), and *S. striatus* (Day, 1867)), the present century has seen the publication of only two taxonomic studies involving the genus from this region (Sreekantha et al. 2006; Praveenraj et al. 2020), neither of which contained molecular data. As such, our phylogeny lacks representation from this region, from which the ancestor of the diversification of *Schistura* on Sri Lanka must necessarily have originated.

The phylogenetic structure and geographic distribution of *Schistura* on Sri Lanka resembles that of *Garra ceylonensis* (Sudasinghe et al. 2021b), which comprise a monophyletic endemic diversification. Both these genera are primarily confined to the wet and intermediate zones of the island and are associated with lotic habitats. Our results show that the monophyly of Sri Lankan *Schistura* is strongly supported in both the mitochondrial and nuclear phylogenies. It appears, therefore, that all populations of *Schistura* on the island derive from a single colonization event by a founder that immigrated at a time when the Palk Isthmus was emergent and supported mesic habitats, probably in the Pliocene, as was the case also in *Garra* (Sudasinghe et al. 2021b). While our study lacks specimens from peninsular India, we think that the monophyly of Sri Lanka *Schistura* will persist even when subcontinental species come to be better represented in molecular phylogenetic analyses.

Mito-nuclear discordance

Our nuDNA phylogeny does not support the monophyly of the mitochondrial lineages recovered by the delimitation analysis. This is not unexpected: the nuclear markers used in this study are relatively uninformative at this phylogenetic scale. Well-resolved and strongly supported mtDNA clades showing clear genetic differentiation among species or lineages are likely to arise more rapidly than nuDNA clades, especially in a diversification across a topographically heterogeneous landscape (Allio et al. 2017; García-Llamas et al. 2018). Because nuclear loci take longer to coalesce into distinct lineages after speciation events (Palumbi et al. 2001), they may show weaker differentiation compared to mtDNA loci, reflecting incomplete lineage sorting or geneflow among recently diverged lineages.

Diversification

Our mPTP mtDNA species delimitation analysis recovered eight distinct evolutionary lineages. Each of these is confined to a single basin or a contiguous group of basins, indicative of robust geographical structure. A similar pattern is reflected also in *Garra*, *Puntius* Hamilton 1822, and *Systomus* McClelland 1838, within which distinct, well-supported lineages show clear geographic structuring (Sudasinghe et al. 2020d, 2021b, 2023b).

The partial incongruence observed between the tree-based mPTP and distance-based ASAP delimitations (Fig. 1) is not unexpected. Despite our extensive sampling of *Schistura* populations across the range of the genus in Sri Lanka (102 samples from 55 sites), these

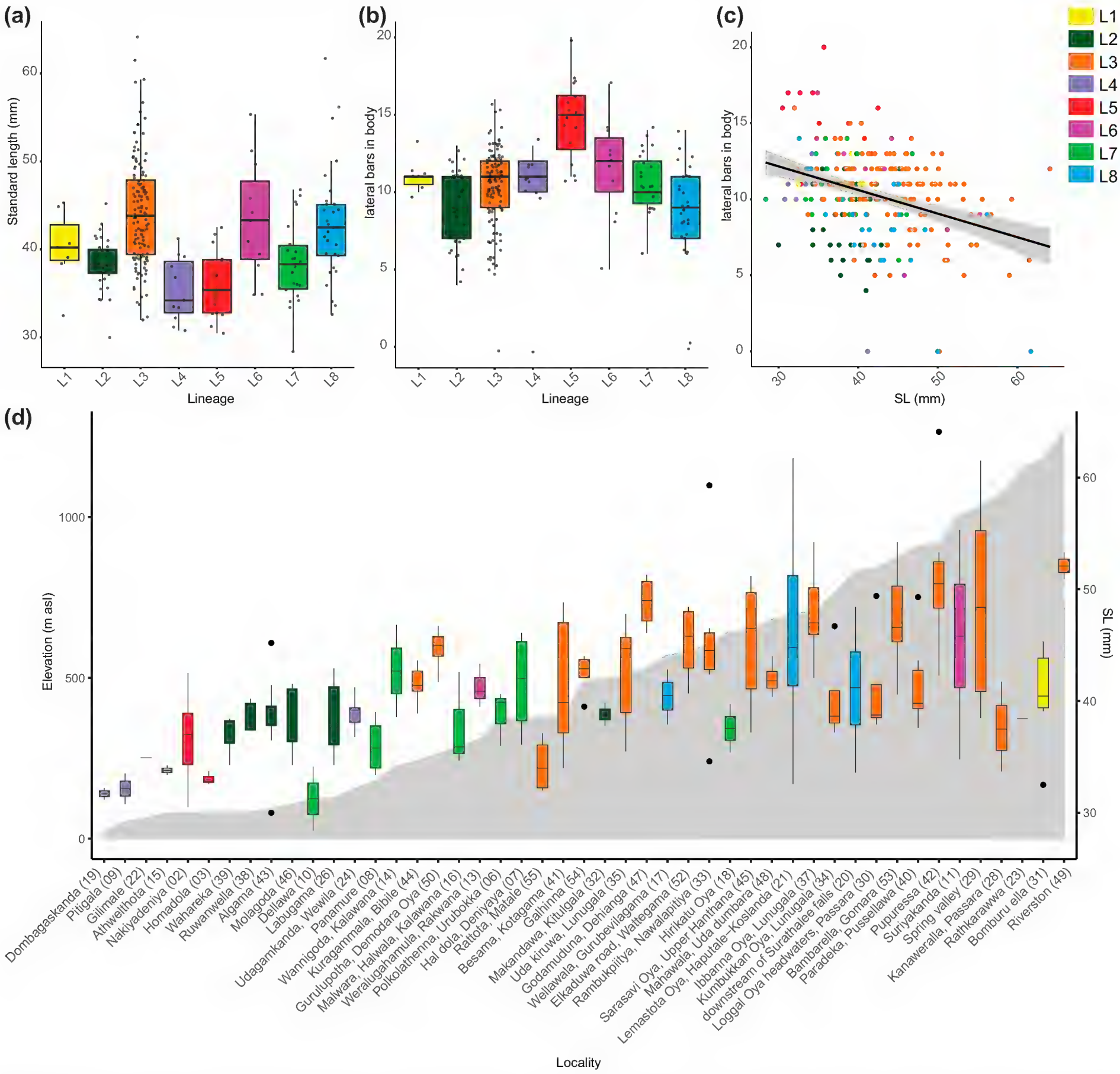

Figure 15. Variation of **A.** Standard length; and **B.** Number of lateral bars in Sri Lankan *Schistura* (n = 261); **C.** Relationship between standard length and number of lateral bars on body; and **D.** Standard length variation along the elevation gradient. Numbers in parentheses represent sampling localities listed in Suppl. material 1.

delimitations necessarily depend on the density of sampling and the coverage of haplotypes: they should therefore be considered open to refinement. The complex topography of the range of *Schistura* in Sri Lanka too, results in spatial structuring and isolation, resulting in maximum intra-lineage genetic distances being underestimated and minimum inter-lineage genetic distances being overestimated (Hubert et al. 2024). Nevertheless, the number of MOTUs recovered by the non-parametric ASAP, which detects barcode partitions, was 9, whereas that recovered by mPTP, a parametric approach, was 8. Despite the similarity of the MOTUs detected by the two methods, the two approaches converged with respect to only three lineages (L1, L6, L8), while ASAP recovered L7 as four lineages.

Taxonomy

For a century-and-a-half from Bleeker (1863) to the description of *Schistura madhavai* and *S. scripta* by Sudasinghe (2017, 2018), Sri Lankan *Schistura* were considered to comprise a single polymorphic species, *S. notostigma* (Pethiyagoda 1991). The delimitation analyses recover *S. madhavai* and *S. scripta* as L6 and L5, respectively, supporting the morphological diagnoses by which these species were originally distinguished. Initially considered to be narrow-range endemics, each confined to a single location, the more extensive sampling of the present study shows them to be more widely distributed.

The identity of *Schistura notostigma* was long in doubt because its type specimens in RMNH (Fig. 2), being

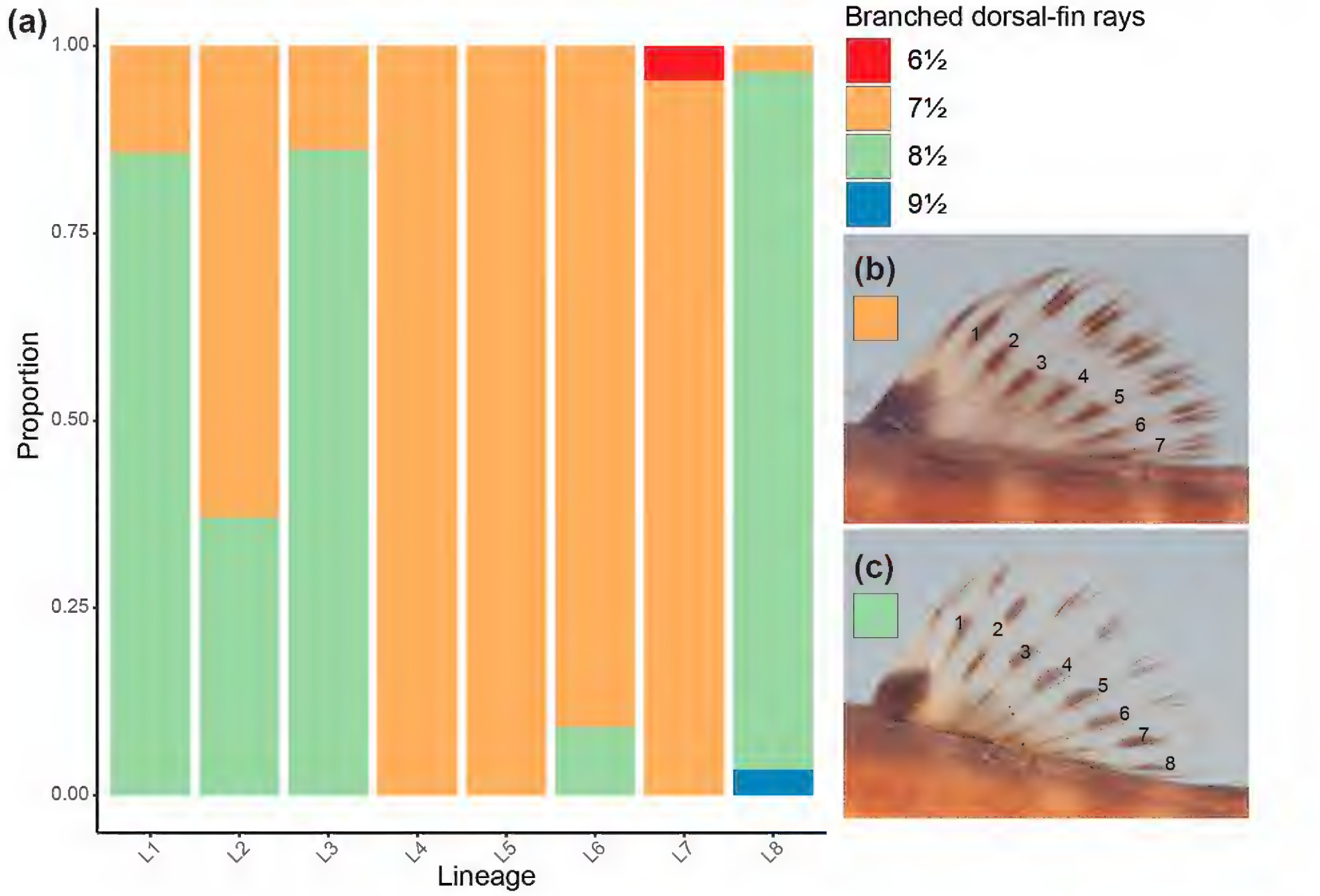

Figure 16. A. Proportionate frequency of the numbers of branched dorsal-fin rays in each lineage of Sri Lankan *Schistura* (n = 261). Representative dorsal fins with **B.** 7.5 and **C.** 8.5 branched rays.

uncatalogued, had never been examined since its original description in 1863. Based on a re-examination of these specimens, Sudasinghe et al. (2025) showed that they differed from *S. madhavai* by possessing 6–7 (vs 8–9) postdorsal bars and having the width of interspaces and bars subequal (vs. interspaces much narrower than bars); and from *S. scripta* by possessing 28–30 (vs 53–76) lateral-line pores, and 6–11 (vs 11–20) total bars on the side of the body. The present data show *S. scripta* (L5) to have an uncorrected pairwise *cox1* genetic distance greater than 3.1% from the other seven MOTUs (cf. an intra-lineage distance < 2.2%), that from *S. madhavai* (L6) being 6.0–6.9% (Suppl. material 5). The uncorrected pairwise *cox1* genetic distance between *S. madhavai* and the other seven lineages is greater than 4.8%. Taken together with their morphological differences, *S. notostigma*, *S. scripta* and *S. madhavai* are valid, clearly distinct species.

The exact type locality of *S. notostigma*, indicated only as 'Ceylon' (= Sri Lanka) in the original description, remains unknown. Based on the morphological characterization of the eight lineages (Suppl. material 8, Figs 15–20), however, the syntypes appear to have originated from a basin in the lowland south-western region of the island (Kelani to Nilwala river basins: L2, L4, L7).

As extensive as our sampling of *Schistura* in Sri Lanka has been, the potential for isolation conferred by the topographical heterogeneity of the island's south-western wet zone is such that finer sampling may reveal yet more distinct lineages in this remarkably biodiverse region (Bossuyt et al. 2004; Pethiyagoda and Sudasinghe 2021). This is evident from the number of distinct genetic lineages observed in (though not confined to) the Kalu and Walawe basins in the south-western wet zone. A total of four (L4, L6, L7, L8) lineages are represented in the Kalu basin and three (L6, L7, L8) in the Walawe basin. However, our sampling of these two basins was not exhaustive (Fig. 1B).

Morphology

The traits included in our study are widely used to diagnose species of *Schistura* (Kottelat 1990). With a few exceptions, the genetic distinctiveness of the eight lineages of *Schistura* in Sri Lanka is only partially reflected in their morphology as observed from the traits examined here. Some traits, however, appear unique to local populations: wider sampling shows these not to extend across the associated mitochondrial genetic lineage. For instance, the population of *Schistura scripta* at the type locality, is easily differentiated morphologically from all congeners by traits such as the number of bars on the body and the number of lateral-line pores. Meanwhile, other populations in close proximity to the type locality cluster genetically with

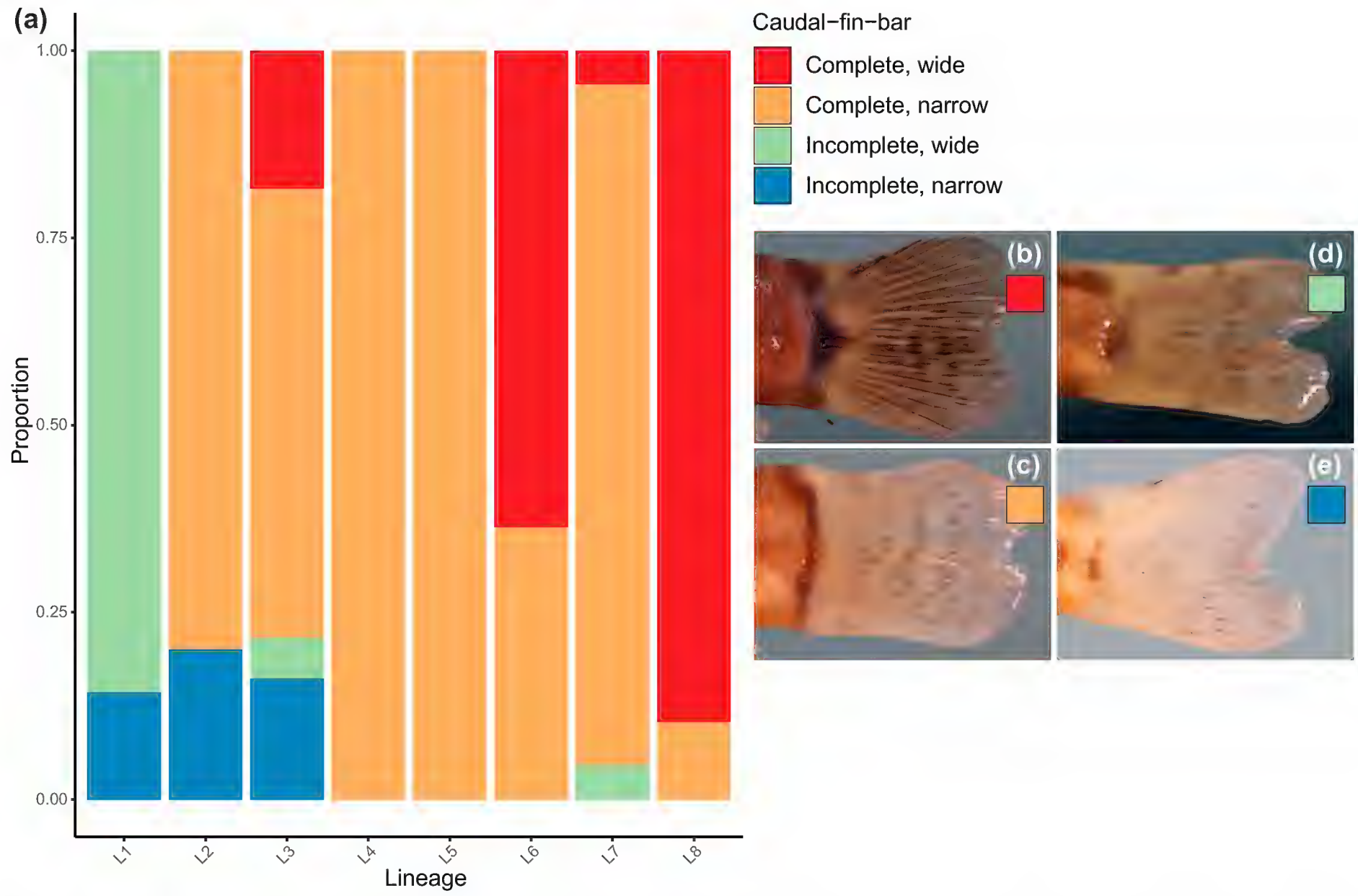

Figure 17. A. Proportionate frequency of the various shapes of the caudal-fin bar in each lineage of Sri Lankan *Schistura* (n = 261). Representative caudal-fin bars that are **B.** Complete, wide; **C.** Complete, narrow; **D.** Incomplete, wide; and **E.** Incomplete, narrow.

S. scripta in L5 but do not consistently exhibit the 'typical' morphological traits associated with this species (Fig. 10).

Similarly, the *Schistura* population sampled at Surathalee Falls (locality 20), which groups with L8, exhibits a strikingly distinct pattern of bars on the dorsum anterior to the dorsal fin (Fig. 13). This coloration is absent, however, in populations sampled at Lemastota (locality 21) and Wellawala (locality 17), which are in close geographical proximity and share the same mitochondrial lineage.

Likewise, the shape of the caudal-fin bar in the population sampled at Bomburu Ella (locality 31) is unique among Sri Lankan *Schistura*: it is incomplete and confined to the medial region of the caudal-fin base (Fig. 17D). In the population of Rathkarawwa (locality 23) in the same basin (Fig. 17E), however, the caudal-fin bar is narrow and incomplete. Both populations, however, group with L1.

Conversely, other traits appear to converge even in disparate genetic lineages. Though lineages L1, L3 and L8, for example, are confined to the highlands, they do not form a monophyletic group (Fig. 1A). Yet, they differ morphologically from lowland lineages L4, L5 and L7, which too do not form a monophyletic group, by their larger size (Fig. 15A, D), having mostly 8½ branched dorsal-fin rays (Fig. 16A), possessing at least a rudimentary pelvic axillary lobe (Fig. 18), and having the scales distinct only on the posterior body (i.e., scales indistinct anterior to the dorsal-fin origin, Fig. 19), possibly also a function of size. Meanwhile, compared to these highland and lowland lineages, L2 appears to exhibit intermediate patterns for these traits.

We suspect that large body size in the highland population is due, at least in part, to release from competition in montane streams, notably depauperate of freshwater fishes (Senthilnathan 2023). *Schistura* are often the only fishes in smaller highland streams, with *Garra ceylonensis*, *Plesiopuntius bimaculatus* (Bleeker 1863) and *Devario* spp. being present in rivers and larger streams. By comparison, Moyle and Senanayake (1984) reported 26 species of freshwater fishes from a stream at Kanneliya, a lowland location.

This discordance between the mitochondrial phylogeny and morphology in Sri Lankan *Schistura* could indicate a rapid diversification following a relatively recent colonization event, with morphological traits not yet fixed within the various lineages. Given the evident topographical complexity, isolation and genetic drift could also play a role in fixing different morphological traits within each population. A similar pattern was observed also in a Sri Lankan cobitid loach, *Lepidocephalichthys thermalis* (Sudasinghe et al. 2023c). There too, although two distinct mitochondrial genetic lineages were recovered, morphological traits overlapped to varying degrees across the two lineages. However, it is important to note that mitochondrial DNA represents only a single, maternally inherited lineage and provides a limited snapshot of evolutionary history. To fully assess the extent and nature of the discordance, it is essential to incorporate informative nuclear genomic data, such as single-nucleotide polymorphisms (SNPs), which can offer a more comprehensive and balanced view of population structure and evolutionary processes.

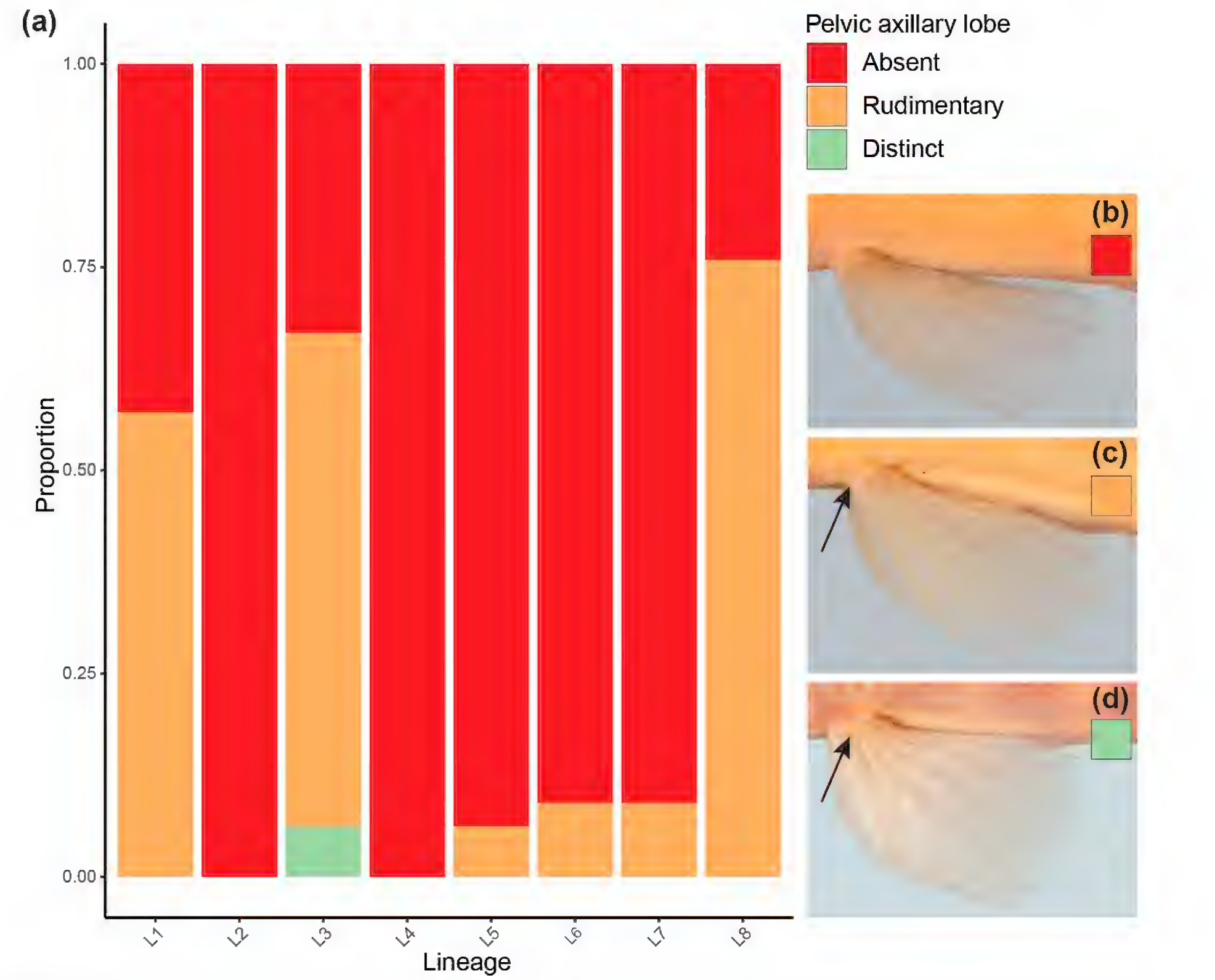

Figure 18. A. Proportionate frequency of presence of a pelvic axillary lobe in each lineage of Sri Lankan *Schistura* (n = 261). Examples in which the pelvic axillary lobe is **B.** Absent; **C.** Rudimentary; and **D.** Distinct.

Phylogeography

Haplotype networks (Figs 6–13) show *Schistura* to exhibit strong philopatry: a phylogeographic structure broadly similar to that observed in *Garra ceylonensis* (Sudasinghe et al. 2021b). Except in L3, haplotypes are nowhere shared between even adjacent basins.

The mitochondrial phylogeny supports a scenario in which the highlands were colonized multiple times via multiple routes. The three exclusively montane lineages (L1, L3, and L8) are paraphyletic. L1 and L8 likely colonized the central mountains through southern basins such as Kalu or Walawe, while L3, the most widespread highland lineage, likely did so via the Kelani basin. This scenario is supported by our results, which revealed a single individual representing L3, a lineage otherwise confined to the uplands of the Mahaweli River basin, that was sampled from Makandawa (location 32) in the Kelani River basin. This suggests a translocation between the Mahaweli and the Kelani, possibly deriving from a headwater capture event. Makandawa is about 10 km from the Kelani-Mahaweli watershed, and while this is the only such case detected in our data set, Sudasinghe et al. (2020c) pointed to a similar instance also in the dwarf snakehead *Channa orientalis*, around the same location. Other putative trans-basin translocations through headwater capture from the Mahaweli to east- and south-draining river systems have also been inferred in *Systomus*, *Laubuka* Bleeker 1859, *Pethia* Pethiyagoda et al. 2012, *Devario* and *Plesiopuntius* Sudasinghe et al. 2023 (Sudasinghe et al. 2020a, 2020b, 2020d, 2021a, 2023b).

The significant and negative results of the neutrality tests for L3, along with its phylogenetic position, supports the hypothesis offered by Pethiyagoda and Sudasinghe (2021) that the Sri Lankan highlands experienced a recent extinction event, probably driven by desiccation, possibly as recently as the Last Glacial Maximum ca. 20,000 years ago. Such a scenario is also reflected in the highland populations of the only other Sri Lankan fishes to occur above an elevation of 1,200 m, *Devario*, *Garra* and *Plesiopuntius* (Sudasinghe et al. 2020a, 2021b, 2023b). In all these cases, based on molecular phylogenies, the most widespread highland lineages are recently derived from lowland ones.

In contrast to L3, L1 appears to be a relictual population that survived at least the last highland desiccation event. Such relictual highland lineages have also been

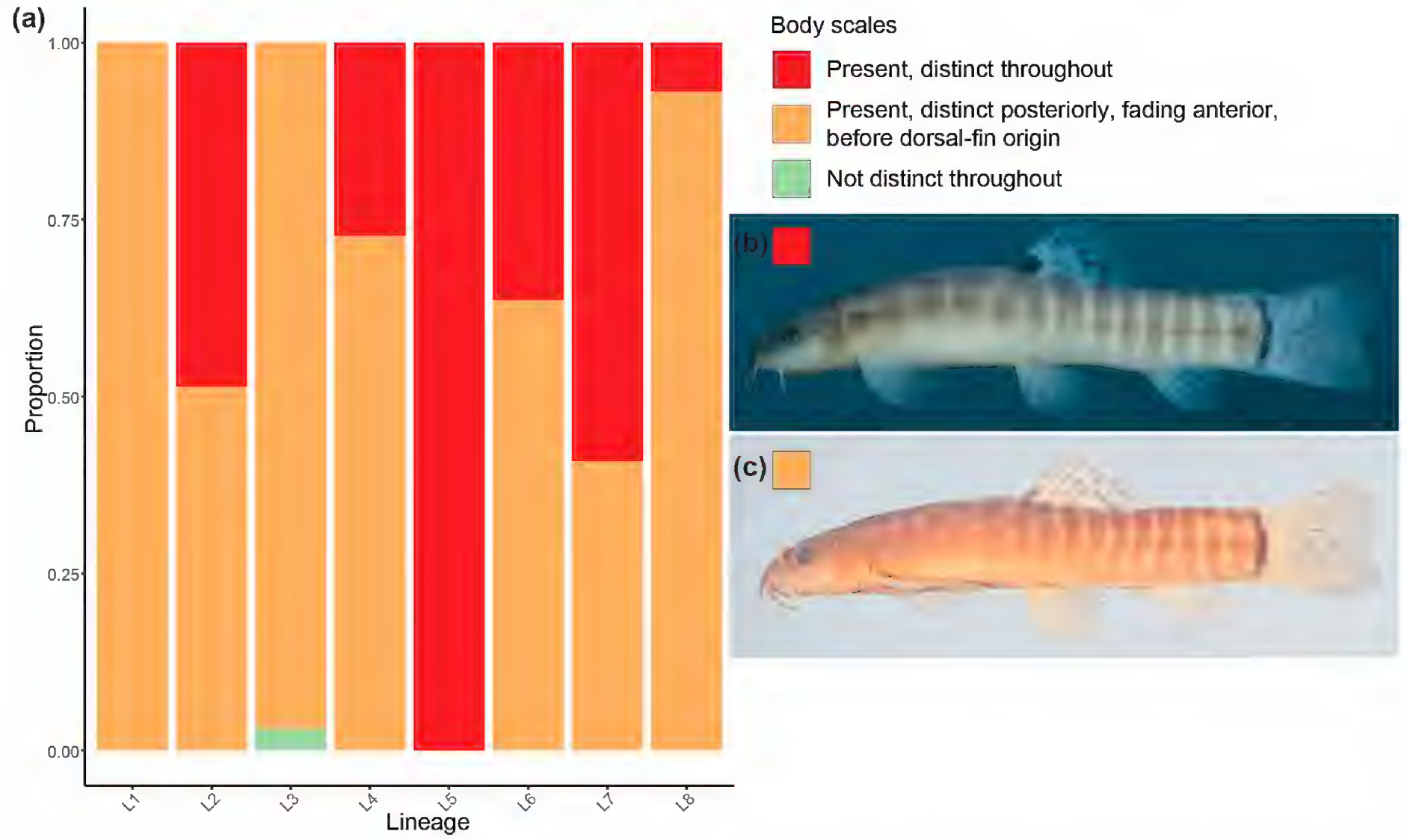

Figure 19. A. Proportionate extent of body scalation in each lineage of Sri Lankan *Schistura* (n = 261). Examples of specimens in which body scales are **B.** Distinct throughout; and **C.** Distinct only posterior to dorsal-fin origin.

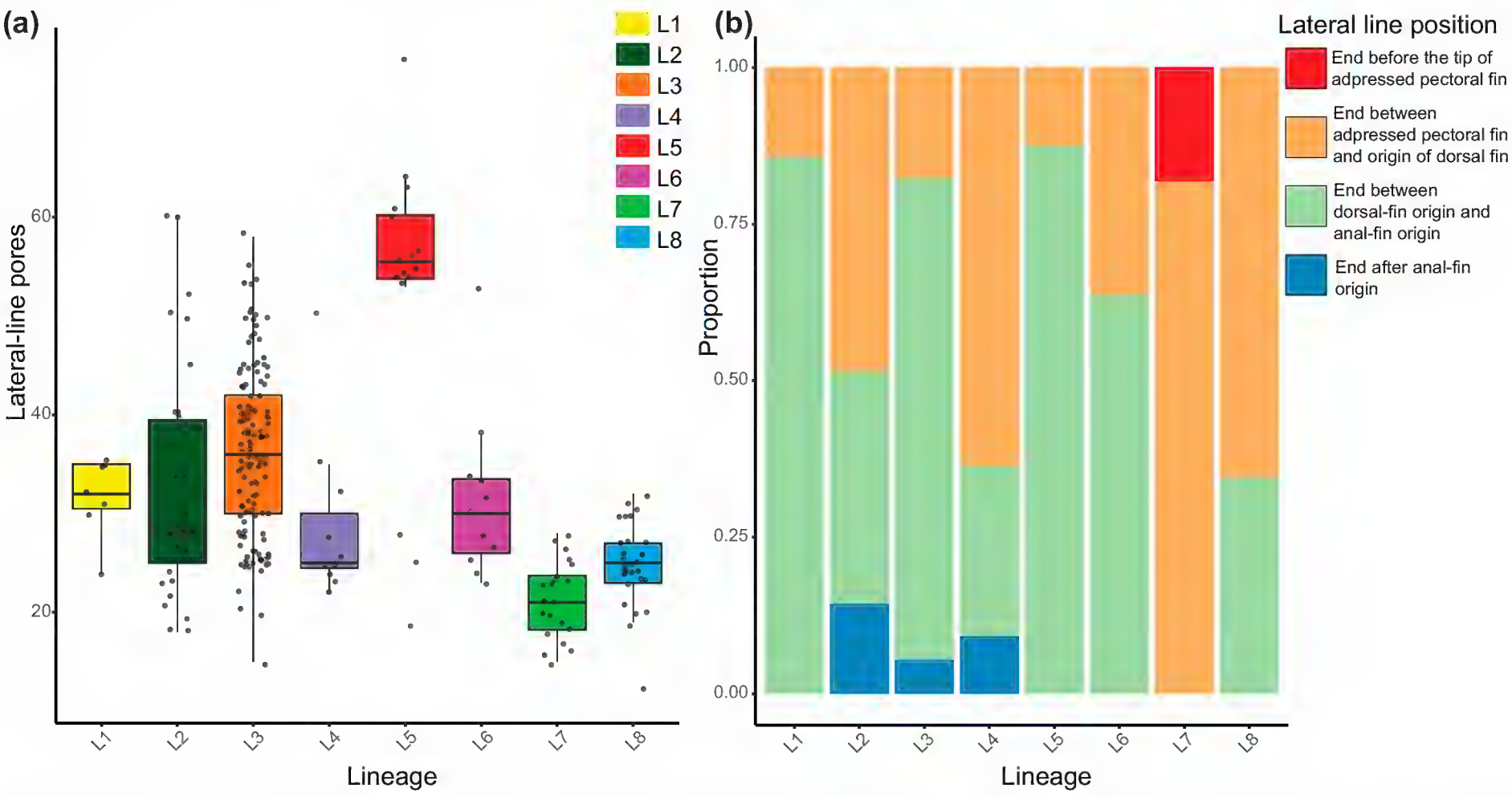

Figure 20. A. Variation in the number of lateral-line pores; and **B.** Proportional extent of the lateral line in each lineage of Sri Lankan *Schistura* (n = 261).

observed in *Garra* and *Devario* (Sudasinghe et al. 2020a, 2021b). It is noteworthy that the Sri Lankan highlands lack specialized hill-stream fish genera such as those that characterize the upland streams of the Western Ghats, such as *Pterocryptis* Peters, 1861, *Indoreonectes* Rita & Bănărescu, 1978, *Balitora* Gray, 1830, *Homaloptera* van Hasselt, 1823, *Bhavania* Hora, 1920, *Travancoria* Hora, 1941, *Barilius* Hamilton, 1822 and *Glyptothorax* Blyth, 1860 (Pethiyagoda and Sudasinghe 2021).

Interestingly, despite the highlands being geographically larger and arguably more topographically complex, the southwestern wet zone lowlands harbor greater genetic diversity (Fig. 1). The southwestern lowlands comprise multiple distinct genetic lineages of *Schistura* within a smaller geographic region. As observed in previous cases such as in *Plesiopuntius bimaculatus*, *Puntius kelumi* Pethiyagoda et al. 2008, *Systomus pleurotaenia* (Bleeker 1863), and *Devario micronema* (Bleeker 1863), the present results

corroborate recent findings that the southwestern basins, especially the Kalu-Gin-Nilwala system, served as a refuge for freshwater fishes and acted as a species pump (Pethiyagoda and Sudasinghe 2021; Sudasinghe et al. 2023b). For example, four lineages are present in the Kalu basin, while only two occur in the Mahaweli basin in the highlands.

Conclusions

Our results confirm the monophyly of Sri Lankan *Schistura*. The relatively homogeneous genetic structure of L3, the most widespread upland lineage, taken together with the significant and negative results of neutrality tests and its phylogenetic position, suggests a recent colonization of the highlands. The occurrence of the same (L3) lineage in the central mountains as well as the Knuckles Massif, which are physiographically discrete and support distinct biotas (Perera and Fernando 2024), adds confidence to this conclusion. The wide distribution of L3 also supports the hypothesis that Sri Lanka's central mountains experienced a recent extinction, possibly as recently as the Last Glacial Maximum, likely driven by desiccation (Prematilake 2012; Pethiyagoda and Sudasinghe 2021). Meanwhile, the restriction of multiple lineages (e.g., L2, L4, L5, L7) to the wet zone lowlands support the idea that the rainforests of this perhumid region may have served as refugia during such events.

Consistent with some previous studies (Sudasinghe et al. 2020a, 2020b, 2020c, 2020d, 2021a, 2023b), our data provide evidence of geneflow between the headwaters of the Mahaweli and the Kelani rivers, as well as between the Mahaweli and southeastern basins such as the Menik, Gal, and Kumbukkan, possibly as a result of headwater capture.

Our results suggest that *Schistura* entered Sri Lanka relatively recently and went on to colonize almost the entirety of the island's mesic south-western quadrant to an elevation of 1,450 m asl, diversifying into eight discrete mitochondrial lineages. This genetic structure, however, is not reflected in the phenetic diversification of the population. Our morphological analysis, which included all 261 preserved specimens of *Schistura* available from Sri Lanka, did not reflect this genetic structure. This decoupling is not unexpected because phenetic variation in rapidly diversifying populations is associated with evolutionary changes in regulatory, rather than structural, genes (King and Wilson 1975; Barrier et al. 2001).

Nevertheless, a substantial portion of new species described from South Asia depend entirely on morphological evidence. Our results show many of the phenetic traits routinely used to discriminate between species in *Schistura* taxonomy to be unreliable in the context of the Sri Lankan diversification. Based on samples from their type localities, the two most recently described Sri Lankan species, *S. madhavai* and *S. scripta*, are morphologically strikingly different from other populations of *Schistura* on the island. But the present study shows that these 'diagnostic' traits disappear when morphological variation within the associated genetic lineages is considered. Indeed, a red flag ought to be raised whenever the words "known only from the type locality" occur in a new-species description.

Ethical statement

Sampling in Sri Lanka was carried out from 2014–2020 under permits issued by the Department of Wildlife Conservation (WL/3/2/59/14) and the Forest Department (R&E/RES/NFSRCM/14-16-4) to HS and MM. The ethics of specimen collection, euthanasia using tricaine methanesulfonate, tissue sampling, and preservation, were approved by the Postgraduate Institute of Science at the University of Peradeniya.

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Author contributions

Conceptualization, methodology: HS, RP and MM; formal analysis, investigation, data curation: HS, KW, TR; writing—original draft preparation: HS; writing—review and editing: KW, TR, RP, MM; project administration: HS, RP and MM. All authors have read and agreed to the published version of the manuscript.

Data availability

Sequence data used in this study are all available via GenBank. All other data that support the findings of this study are available in the main text and supplementary materials.

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Supplementary material 1

Details of sampling localities of *Schistura* for the molecular analysis in the present study

Authors: Hiranya Sudasinghe, Kumudu Wijesooriya, Tharindu Ranasinghe, Rohan Pethiyagoda, Madhava Meegaskumbura

Data type: docx

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Link: https://doi.org/10.3897/zse.101.165370.suppl1

Supplementary material 2

Comparative genetic dataset of Dvořák et al. (2022)

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Data type: docx

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Link: https://doi.org/10.3897/zse.101.165370.suppl2

Supplementary material 3

Specimens of *Schistura* examined in this study

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Data type: docx

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Link: https://doi.org/10.3897/zse.101.165370.suppl3

Supplementary material 4

Measurements, meristics, and traits recorded from specimens of *Schistura* examined for the morphological dataset

Authors: Hiranya Sudasinghe, Kumudu Wijesooriya, Tharindu Ranasinghe, Rohan Pethiyagoda, Madhava Meegaskumbura

Data type: docx

Explanation note: Text.

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Link: https://doi.org/10.3897/zse.101.165370.suppl4

Supplementary material 5

Uncorrected intra- and inter-lineage pairwise cox1 genetic distances among Sri Lankan *Schistura*

Authors: Hiranya Sudasinghe, Kumudu Wijesooriya, Tharindu Ranasinghe, Rohan Pethiyagoda, Madhava Meegaskumbura

Data type: docx

Explanation note: Text.

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Supplementary material 6

Genetic diversity, based on cytb, and cox1, in Sri Lankan *Schistura*

Authors: Hiranya Sudasinghe, Kumudu Wijesooriya, Tharindu Ranasinghe, Rohan Pethiyagoda, Madhava Meegaskumbura
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Explanation note: Text.
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Link: https://doi.org/10.3897/zse.101.165370.suppl6

Supplementary material 7

Component loadings in the principal component analysis of the morphometric measurements and meristics for the Sri Lankan *Schistura* dataset

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Data type: docx
Explanation note: Text.
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Link: https://doi.org/10.3897/zse.101.165370.suppl7

Supplementary material 8

Measurements (mm) and the effect of species and MOTUs on morphological traits for the *Schistura* dataset

Authors: Hiranya Sudasinghe, Kumudu Wijesooriya, Tharindu Ranasinghe, Rohan Pethiyagoda, Madhava Meegaskumbura
Data type: docx
Explanation note: Text.
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Link: https://doi.org/10.3897/zse.101.165370.suppl8

Supplementary material 9

Fisher's Exact Test for categorical traits in Sri Lankan *Schistura*

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Data type: docx
Explanation note: Text.
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